Electrochemical oxidation and cleavage of peptides in bioanalysis

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Mechanistic Aspects & Method Development

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2013

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This work was performed in the research group Analytical Biochemistry and Mass Spectrometry Core Facility of the of the Groningen Research Institute of Pharmacy (GRIP), Faculty of Mathematics and Natural Sciences, University of Groningen, and within the graduate school GUIDE.

The Research presented in this thesis was financially supported by the Dutch Technology Foundation, STW (Grant 07047).

university of groningen

GUIDE

Research Institute for Drug Exploration

Printing by Ipskamp Drukers B.V., The Netherlands

ISBN: 978-90-367-6082-9

RIJKSUNIVERSITEIT GRONINGEN

Electrochemical Oxidation and Cleavage of Peptides in Bioanalysis

Mechanistic Aspects & Method Development

Proefschrift

ter verkrijging van het doctoraat in de
Wiskunde en Natuurwetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
vrijdag 8 maart 2013
om 16.15 uur

door

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geboren op 6 juli 1982

te Phalsbourg, Frankrijk

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A mes Parents,

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Chapter 1

General Introduction

Electrochemistry-Mass Spectrometry

1. Electrochemistry-Mass Spectrometry: Historic overview

The first record of coupling electrochemistry (EC) on-line with mass spectrometry (MS) dates back to the early 1970s when Bruckenstein and Gadde [1] reported the analysis of oxygen that was generated electrochemically on a porous platinum (Pt) electrode by electron impact (EI) mass spectrometry. The technique was however limited to the analysis of volatile species since the porous electrode acted as a permeable membrane in contact with the solution at one side and to the vacuum inlet of the MS at the other side. A decade later, the elaboration of new ionization techniques and progress in the coupling of MS interfaces with liquid sample introduction in the early days of LC-MS allowed the analysis of non-volatile species. In 1986, Hambitzer and Heitbaum for the first time coupled an electrochemical cell on-line with thermospray ionization (TSP) for the analysis of N,N-dimethylaniline [2]. This major breakthrough in the field of EC-MS was rapidly followed by work of several research groups including Brajter-Toth and coworkers for the analysis of purines [3-5] and Getek et al. who studied the oxidation and formation of cysteine (Cys) and glutathione (GSH) conjugates of acetaminophen [6]. The implementation of tandem MS (MS/MS) [3-4] and liquid chromatography (LC) [5] by Brajter-Toth's group constituted a major improvement of the technique since it respectively allowed to obtain detailed structural information and to separate reaction products prior to analysis. The use of commercially available coulometric cells with porous working electrodes having large surface areas was an additional advantage over previous systems since higher flower rates (up to 2 mL/min) could be used resulting in short void times (500 ms) between product formation in the EC cell and product detection by TSP-MS/MS. The early developments of EC-MS have been reviewed by Chang et al. [7] and Volk et al. [8].

The major advantage of EC-TSP-MS was the rapid analysis of reaction products by MS in the gas-phase after extraction of ions from the condensed phase. However, the high temperature applied in TSP-MS could potentially influence kinetics of redox reactions and was not suited for the analysis of thermally labile compounds [8]. Electrospray ionization (ESI) emerged in the 1990s as the method of choice for the coupling of EC and MS. ESI is a soft ionization technique that releases ions from the liquid phase by applying a potential

difference (typically several kV) between a conducting electrospray emitter and the entrance of the mass analyzer yielding minimal fragmentation or decomposition of analytes. The potential applied to the emitter induces electrochemical processes at the liquid-metal interface of the ESI source which can thus be considered as an electrolytic cell as first described by Blades et al. [9]. These inherent electrochemical reactions have been studied in detail notably by the group of Van Berkel [10-14] to gain a better understanding of the electrospray process and are further discussed in chapter 2 (section 2.3.2). Bond et al. [15], who designed a simple two-electrode system for the analysis of metal-diethyldithiocarbamates, and Van Berkel and coworkers [16], who presented three different setups, pioneered the coupling of EC and ESI-MS. Lu et al. [17] published the first study using on-line linear sweep voltammetry in 1997 which allowed the construction of mass voltammograms that follow product formation versus applied potential and give detailed information on reaction mechanisms occurring at different potentials.

Although EC has been combined with several other ionization sources such as fast atom bombardment (FAB) [18], particle-beam (PB) [19,20] and APCI [21], coupling with ESI-MS remains by far the most widely used. The possibility to setup integrated, automated systems, facilitating high-throughput applications is a clear advantage of the on-line coupling of EC-MS and is of major interest for research fields such as the study of drug metabolism, metabolomics and proteomics. As mentioned earlier, the ability to observe short lived species and/or reaction intermediates on integrated systems is another appealing feature of EC-MS. Reaction products can be produced in small-volume cells (µL range) which, when connected to the inlet of the MS, allow detection in a few seconds or less [22-26]. Miniaturization of electrochemical cells and transfer of the technique onto a chip is the latest technological breakthrough in the field. On-chip electrochemistry is particularly interesting when only small sample volumes are available and provides very short transfer times to the MS detector when the chip is equipped with an ESI emitter [27]. Fully integrated systems on-chip containing an EC cell, LC separation and the ESI emitter are conceivable and may solve interfacing problems. Applications have been reported in the fields of electrosynthesis [28] and drug metabolism [29,30]. The development of automated systems [31,32] has shown promising results which will undoubtedly be followed by many

others in the near future. Early developments of electrochemical techniques for lab-on-a-chip applications have been reviewed by Nyholm [33].

2. Practical aspects of EC-MS

The coupling of EC to ESI-MS is fairly easy to setup. However, the combination of both techniques comes with some restrictions and practical aspects need to be considered in order to make both methods fully compatible. The ESI high voltage, which induces upstream currents, the solution composition and the EC cell design are points that require special attention and are discussed here briefly.

2.1 ESI high voltage and upstream currents

The design of ESI mass spectrometers varies among different manufacturers. The high voltage necessary for the ESI process can, for instance, be applied either at the entrance of the MS (in which case the ESI emitter is grounded) or at the ESI emitter itself. The latter configuration is the most frequently implemented and generates electrochemical reactions at the liquid–emitter interface (see section 2.3.2). Electrochemical reactions inherent to the ESI source induce currents flowing between the tip of the ES needle and the EC cell if the latter is grounded. These currents can affect electrochemical reactions occurring within the EC cell but can also damage instrumentation placed upstream to the ESI source (e.g. the LC system). The EC system and any other upstream instruments must thus be decoupled in some way. Placing a grounding point between the EC cell and the emitter or in some cases a long transfer line (~30 cm) will allow decoupling of the backwards currents. A long transfer line will increase delay between EC and MS and electrochemically generated products may not be detected depending on their lifetime. A grounding point however will act as a counter electrode where electrochemical reduction or oxidation reactions will occur when operating the ESI source in positive or negative mode, respectively. These products can also influence electrochemically generated species in the EC cell and one should be aware of such possibilities. Floating the EC device at high voltage is a more preferable way to decouple the cell from the mass spectrometer high voltage [16]. A battery-operated potentiostat and/or an isolation transformer are used to float the EC cell at the high voltage of the ESI

emitter. Alternatively, controlled-potential electrochemistry (CPE) can be achieved by implementation of two- or three-electrode systems (including porous flow-through (PFT) electrodes for high conversion) within the ESI source. This allows precise control of ESI-inherent electrochemistry and prevents additional or uncontrolled reactions in the emitter [17,34-38].

In all experiments described in this thesis, a grounding point was placed between the ESI source and the upstream instruments together with long transfer lines to avoid interference due to backwards currents.

2.2 Influence of solution composition

Another restriction and compatibility issue that emerges when coupling EC-MS on-line is the supporting electrolyte required for EC which is in many cases incompatible with MS. The yield of EC reactions is low if the conductivity of solutions in water or organic solvents is low. In order to obtain good conversions within the EC cell, addition of electrolytes is necessary to increase solution conductivity and decrease the ohmic potential drop causing the electrode potentials to deviate from the potential imposed by the potentiostat. Buffer salts are widely used in electrochemistry but lead to ion suppression, signal saturation, or plugging of the MS orifice and are not compatible with MS analysis. A careful choice of supporting electrolyte must thus be made and priority should be given to volatile solvent additives that are ‘MS-friendly’. Control of the solution pH is another crucial aspect that requires special attention since protonation or deprotonation of the targeted analytes alters the outcome of the electrochemical reaction. Ammonium acetate or ammonium formate salts are recommended for aqueous solutions. Dilute acetic or formic acid (low pH) and ammonia (basic pH) can be used either alone or added to ammonium acetate/formate salts depending on the desired pH. Ammonium-based electrolytes or ammonia, however, have the potential disadvantage of reacting as nucleophiles with the electrochemically generated intermediates, forming unwanted products. Jurva et al. [39] have listed a number of suitable alternative electrolytes for the oxidation and detection of lidocaine in an on-line EC-MS setup. Lithium trifluoromethane sulfonate (i.e. lithium

triflate) has been suggested as a compatible electrolyte when working in organic solvents [13].

In conclusion, a compromise has to be made to accommodate both techniques depending on the desired electrochemical reaction. Moreover, implementation of an LC separation step between the EC device and the MS may allow separating the supporting electrolytes from the analytes of interest and broaden the range of useable electrolytes.

2.3 EC cell design

Although several home-made EC cells have been developed for specific purposes, commercial flow-through cells are more widely used for on-line EC-MS applications. The latter are found either as thin-layer cells or cells with a porous working electrode. Both thin-layer and porous flow-through (PFT) electrochemical cells have been used for the studies described in this thesis and both designs come with their benefits and shortcomings.

PFT electrodes are characterized by their large surface area and yield high analyte conversion at high flow rates (up to several hundreds of µL/min for synthesis cells). Some cells are designed to withstand high pressures and can thus be placed in front of an HPLC column. The coulometric 5021 conditioning cell of ESA was used in the majority of the experiments described in this thesis. It contains a large porous graphite electrode and palladium auxiliary and reference electrodes that are located upstream in the flow path from the working electrode. The major shortcoming of the PFT coulometric cells manufactured by ESA is the fact that they cannot be disassembled for cleaning or replacing the electrodes. Cell history is therefore an important factor influencing the performance of the cell, although cleaning procedures with diluted acids can help to regenerate the surface to some extent.

Thin-layer electrochemical cells have small surface areas, typically estimated to be 50 times (or more) smaller than PFT coulometric cells. A direct consequence is that much lower flow rates have to be used to reach adequate electrochemical conversions. The volume of the cell is defined by a spacer that is placed between the working and the auxiliary

electrode and can thus be adjusted to fit specific purposes (e.g. a thin spacer for analytes with low diffusion rates). The proximity of the auxiliary electrode to the working electrode is a drawback of thin-layer cells. Reactions occurring at the auxiliary electrode are known in EC to potentially affect the outcome of the desired electrochemical reaction by either reacting with products generated at the working electrode or by appearing as additional peaks in the mass spectra which can lead to misinterpretation. The positioning of auxiliary and working electrodes inside the coulometric PFT cell minimizes such unwanted interferences and is thus a clear benefit of this cell. Thin-layer cells can, unlike coulometric cells, be disassembled and the working electrodes can thus be regularly checked, cleaned and mechanically polished to ensure surface regeneration.

3. Applications for EC-MS

A couple of selected applications where EC-MS has proved useful are described here to show the potential of the technique. Advances and developments in the field of EC-MS have been reviewed over the past years [40-46].

3.1 Electrochemically assisted ionization

Ionization of compounds with electrospray relies on the presence of suitable functional groups that can be protonated or form adducts with metal-ions in order to produce ionized species in solution. When such functional groups are not present, the process known as Electrochemically Assisted Ionization (EAI) can either generate charged species directly or produce functional groups that can further be ionized in solution. Formation of radical cations and chemical derivatization of aromatic and highly conjugated molecules [10,11,13,47,48] but also of alkenes and alkynes [49] are examples where ionization of initially neutral analytes was achieved by on-line EC-MS. Introduction of oxygen via S-oxidation reactions of dibutylsulfide [50], phenothiazine [51] or epoxidation of benzopyrene [50] generated functional groups that could be protonated and subsequently detected by MS. An alternative method, introduced by Van Berkel and coworkers, consists in synthetizing ferrocene-based electrochemically ionizable derivatives that allow or enhance ESI-MS analysis of a variety of compounds [34,52]. A range of non-polar alcohols, sterols and

phenols was successfully derivatized and detected by MS thanks to the oxidation of the Fe^{2+} to the Fe^{3+} ferrocenium ion, by taking advantage of the electrolytic process inherent to the operation of the ESI source in positive mode. The method provides stable, preformed ions in solution and ensures increased sensitivity in MS analysis even for compounds that can be protonated. More importantly, it also broadens the range of compounds amenable to ESI-MS analysis [52]. Diehl et al. assembled a hyphenated LC-EC-MS system where a mixture of ferrocene-derivatized alcohols and phenols was analyzed [53]. The derivatized analytes were separated on an LC column prior to oxidation of the ferrocene and detection in the MS. The same group extended the derivatization method to the analysis of isocyanates [54] and thiols [55,56]. Ferrocene-based derivatization has been reviewed recently by Seiwert et al. [57].

3.2 Electrochemical tagging

EC occurring at the ES-emitter of microfluidic chips has been exploited by Girault and coworkers for the tagging of peptides and proteins by means of quinone addition [58-63] (see also section 2.3.2 in chapter 2). The tagging strategy consists of oxidizing hydroquinone to benzoquinone which subsequently reacts specifically with free Cys residues in peptides and proteins. The reaction was shown to follow an ECE (Electrochemical-Chemical-Electrochemical) mechanism since the reduced conjugated quinone is almost instantly re-oxidized in the ESI source [58,59]. The incorporation of functional groups onto hydroquinone precursors was shown to have a significant influence on tagging yields and substituted electron-withdrawing groups gave improved yields [60]. Up to three Cys residues could react within the same peptide [62] and successful tagging was shown to take place even for intact proteins although slower kinetics and steric hindrance affected the yields [61]. The technique can be used as a method for the “on-line counting” of Cys residues within peptides generated after enzymatic digestion [62]. Knowing the frequency of a specific amino acid within a peptide is valuable information for protein identification through database search [64,65]. Van Berkel et al. extended the method to the tagging of disulfide linked peptides [66]. A PFT electrode placed at the upstream grounding point of the ESI source successfully reduced the disulfide bridge

providing two free Cys residues that underwent conjugation with oxidized hydroquinone at a second PFT electrode placed at the emitter tip. Girault and coworkers also introduced the use of copper, zinc, nickel, iron and silver sacrificial electrodes to generate their corresponding metal ions in solution for further reactions with peptides and proteins [67]. Copper ions were used to study copper-Cys interactions [68-70] that are known to promote inter- and intra-molecular disulfide bridging [71], whereas zinc ions where shown to successfully react with tyrosine- and serine-phosphorylated peptides [70,72].

3.3 EC-MS in drug metabolism research

Drug metabolism consists in simple terms of the absorption, distribution, interaction with targets, and eventual elimination of xenobiotic compounds from the body. Drug metabolism can be divided into two phases. Phase I metabolism aims at converting xenobiotic compounds into more polar derivatives by unveiling or introducing functional groups (e.g. oxidation, reduction, hydrolysis) while phase II metabolism conjugates the polar functional groups of phase I metabolites with endogenous compounds (e.g. glucuronic acid, sulfates, GSH, amino acids) to facilitate their excretion. *In vivo* Phase I metabolism is performed by a variety of enzymes, the most studied of which constitute the cytochrome P450 superfamily (CYP450). CYP450 monooxygenases cover a wide range of biotransformations and are able to convert a large variety of substrates [73,74]. CYP450-catalyzed reactions proceed via two principal mechanisms based on single electron transfer (SET) and hydrogen atom transfer/abstraction (HAT) oxidation reactions.

An important part of EC-MS studies aims at mimicking *in vivo* drug metabolism. Prediction of the metabolic fate of potential new biologically active compounds in the early stage of drug development is of crucial importance to determine the range of active, inactive or toxic, non-toxic metabolites that may be generated from a specific drug. Conventional methods to study metabolic conversions include preclinical *in vivo* studies in laboratory animals and *in vitro* experiments with liver cells, subcellular fractions from animal or human liver tissue (e.g. microsomes, cytosol) containing enzymes in their natural environment which allow formation of the full range of metabolites. These methods generate, however, complex mixtures and intensive purification steps may be required to

detect and identify the metabolites. *In vitro* drug metabolism studies by electrochemistry are thus an alternative and complementary approach to these conventional methods and have the potential to produce metabolites in sufficient purity and quantity for subsequent *in vivo* toxicity studies. The ability of fast screening and high throughput is an additional benefit of EC-MS over the other techniques and can prove useful for a rapid and detailed evaluation during the early development stages of drug candidates. A brief overview of the current state of EC-MS in drug metabolism research will be presented with a focus on selected studies in relation to CYP450 reactions highlighting the developments in the field. More detailed information can be found in recently published reviews [43,45,46,75,76].

3.3.1 Direct electrochemical oxidation

Direct electrochemical oxidation at unmodified electrodes (usually carbon) is the most straightforward technique to generate metabolites based on well-known reactions from organic electrochemistry [77]. Getek et al. pioneered the use of EC-MS in drug metabolism research more than 20 years ago. They studied the oxidation of acetaminophen on a glassy carbon electrode showing the formation of the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) by a dehydrogenation reaction [6]. The potential of EC-MS was further demonstrated by mimicking phase II metabolism reactions as they successfully observed conjugation of Cys and GSH with NAPQI by simple addition of the reactants with a mixing tee placed downstream of the EC cell. A major development in the field was achieved by Jurva et al. when they carried out a systematic study to evaluate the extent of CYP450 reactions that can be imitated by direct electrochemical oxidation [78]. The study of lidocaine showed that N-dealkylation reactions could be performed whereas O-dealkylation of 7-ethoxycoumarin was not observed. They further demonstrated the potential of direct electrochemical oxidation to yield S-oxidation, P-oxidation, alcohol oxidation, and dehydrogenation products [50]. Based on these results, it was concluded that direct oxidation only produces metabolites resulting from reactions that follow the SET mechanism with the condition that they do not require oxidation potentials greater than the oxidation potential of the solvent (usually water). Metabolites that were formed by reactions following a HAT mechanism, such as O-dealkylation, alcohol and aldehyde oxidation to

carboxylic acids as well as hydroxylation of non-activated aromatic rings, were not observed [50].

Several applications were developed in the following years which further contributed to reveal EC-MS as a promising new tool for metabolism studies. Mimicking of phase II metabolism reactions, notably by conjugation with GSH, allowed the correlation of a wide range of conjugates to metabolites observed by the conventional *in vitro* and *in vivo* approaches [79-81]. Transient species, undergoing fast degradation, can be trapped as well, as shown by Karst and coworkers for the study of clozapine. The reactive nitrenium ion, formed upon metabolic activation of clozapine, was successfully trapped by GSH and detected by LC-MS analysis [82]. The same group presented an original setup in which the reactor for the generation of metabolites was alternatively an EC cell or a reactor containing the enzyme horseradish peroxidase (HRP) chemically immobilized on magnetic microparticles [83-85]. Both biomimetic models were compared to microsomal incubation experiments and proved to be suitable for simulating the metabolism of amodiaquine, amsacrine and mitoxantrone [83]. Another variation in the setup showed the implementation of a reaction coil placed between the EC cell and the HPLC column. The addition of glutathione-S-transferase (GST) and a GSH solution to the EC effluents permitted the enzyme-catalyzed conjugation reactions of toremifene [84]. Last but not least, conjugation reactions of electrophilic metabolites of paracetamol, amodiaquine and clozapine (nitrenium ion) were observed to occur with the free Cys thiol groups of β-lactoglobulin A and human serum albumin [81,85].

Recently, Nouri et al. introduced a novel approach for the selective generation of metabolites of lidocaine [86] and phenacetin [87] by square-wave potential pulses on a Pt electrode. Varying the pulse cycle time at the Pt surface promoted reactions that are not feasible with direct, constant potential electrochemical oxidation. Short cycle times (< 0.1 s) yielded the N-dealkylation metabolite while long cycle times (1 s up to 100 s) produced lidocaine-N-oxide by reaction with electrogenerated hydrogen peroxide (under air atmosphere) [86]. Intermediate cycle times (between 0.2 s and 12 s) promoted the formation of 4-hydroxylidocaine. The pulses presumably allow regeneration of the passivated Pt

electrode surface during the low potential step of the pulse. Although the underlying mechanisms for the selectivity of N-dealkylation and 4-hydroxylation remain to be elucidated, a different orientation of the substrate towards the electrode surface is believed to play a role.

The latest innovative approach in the field of EC-MS was introduced by Falck and coworkers who, for the first time, implemented an EC/LC/MS setup combined with a protein kinase binding assay to assess the biological activity of electrochemically generated drug metabolites [88]. The integrated approach was developed for the oxidation, identification and biological characterization of p38α mitogen-activated protein kinase inhibitors. Fluorescence enhancement by competition of the oxidation products with a tracer upon binding to the orthosteric site of p38α kinase was used as the biological readout. Assessing the biological activity of drug metabolites is of great importance for new chemical entities. Implementation of a biological assay provides thus an additional valuable feature to the conventional EC-LC-MS setup.

3.3.2 EC-mediated chemical reactions

Due to the inability of direct electrochemical oxidation to mimic the entire range of biotransformations achieved by the CYP450 enzyme family, several electrochemically assisted techniques have been introduced to mediate the production of additional oxidative drug metabolites e.g. via HAT reactions. Electrochemistry can be used to produce reactive oxygen species (ROS) either by catalytic activation using iron cations or by direct activation of molecular oxygen. The Fenton reaction (Eq. (1)) produces hydroxyl radicals by the homolytic activation of hydrogen peroxide in the presence of ferrous ions (Fe^{2+}). Ferric ions (Fe^{3+}) are electrochemically reduced to Fe^{2+} which subsequently reduce hydrogen peroxide to produce hydroxyl radicals and hydroxyl anions. Only catalytic amounts of ferric ions are required since they are regenerated during the reaction.

Fenton Reaction: $$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \quad (1)$$

Hydroxyl radicals have a broad range of reactivity and react readily with many substrates via electrophilic/nucleophilic additions or HAT mechanisms. Aromatic hydroxylation of benzoic acid at various positions by an EC-induced Fenton reaction was shown for the first time by Oturan et al. [89]. Jurva et al. successfully trapped the generated radicals by the radical scavenger DMPO (5,5-dimethyl-1-pyrroline-N-oxide) and showed oxygen atom insertion reactions into organic compounds that were not observed by direct electrochemical oxidation [90]. The EC-Fenton reaction was also used by Johansson et al. to simulate the most relevant metabolic reactions with different EC-MS systems [91] and by Bruins and coworkers to expand the range of lidocaine metabolites [92].

Alternatively, hydroxyl radicals can be generated by oxidation of water on boron-doped diamond (BDD) electrodes if sufficiently high potentials are applied. BDD electrodes, which are described in chapter 5 of this thesis, have become popular materials in electrochemistry for both direct electrochemical oxidation and for their ability to induce indirect oxidation by formation of ROS. Hydroxyl radicals are generated only at pH values lower than 9 [93] and have been used for the oxidation of hydroquinone, resorcinol and catechol [94].

Nouri et al. recently presented an original method for the generation of ROS by controlled electrochemical reduction of molecular oxygen on a gold electrode [92]. A two-compartment cell was used with the goal to isolate and separately analyze the products formed at the working (cathode) and auxiliary (anode) electrodes. Cyclic voltammetry experiments and structure elucidation by LC-MS/MS showed that direct oxidation of lidocaine at the anode yielded the N-dealkylation metabolite while the N-oxide was produced at the cathode by ROS intermediates after reduction of molecular oxygen.

Electrode modification is another promising approach for mimicking *in vivo* metabolism using electrochemistry. Modifications with different types of porphyrins and porphyrin analogs have been performed to promote a variety of redox reactions in an attempt to mimic the active center of CYP450 enzymes [95-101]. Immobilized metalloporphyrins allow for more selective reactions and provide a wider range of metabolites compared to direct electrochemistry methods. However, the lack of a

surrounding protein environment prevents regioselective and stereoselective reactions to occur. Different immobilization methods of metalloporphyrins have been described [102,103] and can influence their reactivity. The use thiolate ligands to coordinate with the metal center can for instance emulate the binding to Cys in CYP450 [104]. Alternatively, enzymes themselves can be bound to electrodes which have the advantage over metalloporphyrins of selectivity for certain substrates and reaction pathways due to the protein environment surrounding the active site [105]. Peroxidase containing heme prosthetic groups [106,107], synthetic heme peptides [108] and CYP450 enzymes [109,110] have been studied and shown to have potential in terms of promoting selective reactions. Electrode-bound enzymes are promising tools for drug metabolism research that may be used in combination with LC-MS for rapid screening of drug candidates.

3.4 EC-MS in protein analysis

The present section gives an overview of applications where EC-MS was employed for protein analysis. The benefits of EC-MS over conventional techniques include speed of analysis and the possibility to avoid laborious enzyme handling, long incubation times and/or purification and extraction steps. This section will regularly refer to chapter 2 of this thesis where oxidative protein labeling techniques and applications of MS-based proteomics have been reviewed in more detail [111].

The study of protein oxidation is of major importance in biological processes and has been associated with a wide variety of diseases and with ageing [112]. Mimicking *in vivo* reactions helps understanding the underlying mechanisms and can be used to produce oxidized proteins for further research. Protein oxidation *in vivo* occurs either via reactive agents or by direct oxidation of specific amino acid side chains. ROS are the most common reactive agents formed *in vivo* and are derived from the environment (e.g. from cigarette smoke) or from biological processes such as the mitochondrial production of ATP or during inflammation. These oxidation agents and their means of production are listed in section 2.1 and section 2.2 in chapter 2. Direct oxidation reactions occur with the sulfur-containing cysteine (Cys) and methionine (Met), and the aromatic tyrosine (Tyr), tryptophan (Trp) and

histidine (His) side chains. Both indirect, ROS-induced, and direct oxidation reactions can be performed *in vitro* using electrochemical methods to mimic different *in vivo* mechanisms.

3.4.1 Peptide and protein cleavage

Identification and characterization of proteins relies on their residue-specific cleavage into a set of smaller peptides. Peptide masses obtained by MS analysis and further sequence information by MS/MS fragmentation can be compared with *in silico*-generated spectra using database search algorithms in order to identify the proteins of interest. Enzymatic digestion is the method of choice for peptide cleavage (e.g. trypsin digestion) but alternative methods such as chemical cleavage are used when enzymes do not have the required specificity or reactivity. Electrochemical cleavage is another alternative, and purely instrumental, protein digestion method.

Almost 50 years ago Witkop and coworkers [113,114] and Farber et al. [115] reported the first observation of an electrochemical cleavage of peptides next to Tyr residues at Pt electrodes. The cleavage reaction was shown to occur in a similar way as the chemical cleavage with brominating and iodinating oxidative agents [116-118]. Although these innovative experiments were presented as holding great promise, further developments were rather limited. Improvement of MS detection techniques and further developments in protein analysis resulted in renewed interest for this technique in the late 1990s. MacDonald and Roscoe [119] reported the electrochemical cleavage next to Trp residues in dipeptides and confirmed the already observed cleavage next to Tyr at Pt electrodes. The same year, Walton et al. observed coincidentally the electrochemical cleavage of the peptide bond between two Trp residues in hen egg-white lysozyme while investigating the electrochemical nitration of proteins [120]. The latter publication is the first report of electrochemically induced protein cleavage. Permentier et al. followed this up by studying the electrochemical oxidation and cleavage of peptides [121] and proteins [122] in more detail providing a better insight into the benefits and shortcomings of the technique. They used both online EC-MS and EC-LC-MS setups to detect and characterize electrochemically generated cleavage and non-cleavage products. The study of various peptides (< 2.5 kDa) showed that cleavage occurred at least to some extent within all Tyr-containing peptides.

Construction of mass voltammograms relating the applied potential to product formation indicated that side reactions and dimer formation are initiated at high potentials. More importantly, phosphorylated Tyr residues did not undergo cleavage reactions which makes the technique suitable to distinguish between phosphorylated and unphosphorylated Tyr in proteins [121]. This constitutes an important benefit since phosphorylation plays an important role in *in vivo* regulatory mechanisms and the activation or deactivation of many enzymes [123]. A follow-up study examined the oxidation and cleavage of a set of proteins of various sizes. Cleavage C-terminal to almost all Tyr and Trp residues was observed for the small proteins insulin and α-lactalbumin (6 and 14 kDa) while the ratio of cleaved vs. uncleaved sites decreased with increasing protein size [122]. Several proteins did not yield cleavage reactions indicating that electrode surface accessibility of Tyr and Trp residues and competing oxidation reactions were detrimental for the cleavage of large proteins. Prior reduction of the disulfide bridges of lysozyme facilitated cleavage reactions due to improved accessibility of Tyr and Trp residues. Investigation and optimization of experimental parameters is addressed in chapter 3 where the range of cleavage and non-cleavage products has been studied in more detail [124]. The influence of vicinal amino acid, electrode potential, pH and supporting electrolyte was investigated in both on-line EC-MS and EC-LC-MS setups.

The authors also pinpointed adsorption issues of peptides and proteins on the surface of the porous graphite electrodes which is among the major shortcomings of the method since it can impair repeatability and reproducibility, or even prevent oxidation to occur at all. Boron-doped diamond (BDD) has emerged as a promising alternative electrode material for organic electrochemistry notably due to its improved properties in terms of adsorption. Evaluation of a BDD electrode for the oxidation and cleavage of peptides is detailed in chapter 4 of this thesis.

A recent study of Basile and Hauser combined the EC-LC-MS setup with an on-line microwave heated acid hydrolysis flow cell [125]. The shortcomings of both microwave acid hydrolysis at aspartic acid (Asp), which yields long peptides due to the scarcity of Asp in proteins, and the decreasing EC cleavage yields obtained with large peptides and proteins

(< 4 kDa) were addressed. Microwave hydrolysis of proteins at Asp produced peptides with an average length of 16 amino acid residues, which are not very suitable for collision-induced dissociation (CID) fragmentation in MS/MS analysis. The combined microwave/EC method however yielded peptides with an average length of 10 amino acids which is similar to the average of 9 amino acids after tryptic digestion. As a result, the peptides that were produced by this novel non-enzymatic cleavage method produced protonated species with mostly singly and doubly charged ions after ESI which are suitable for MS/MS analysis. The combination of both methods presents advantages in terms of speed of digestion and operation without reagents that are incompatible with downstream ESI-MS analysis. Acid hydrolysis at Asp can for instance be performed with volatile organic acids such as formic or acetic acid that can also serve as electrolyte for EC cleavage, which is more efficient at low pH as shown in chapter 3 of this thesis [124]. Moreover, microwave heating can induce reduction of disulfide bridges when dithiothreitol (DTT) is added to the mixture, which allows for more efficient acid hydrolysis at Asp and EC cleavage at Tyr and Trp. The combined microwave/EC-LC-MS setup has been successfully applied for the digestion of a series of test proteins and a protein extract from an *E. coli* cell lysate. The method has a total experiment time of 6 min and provides good sequence coverage allowing protein identification with a high degree of confidence demonstrating a notable improvement over either non-enzymatic digestion technique alone but still lagging behind enzymatic digestion.

3.4.2 Electrochemical cleavage of disulfide bonds

Cleavage of disulfide bonds is required for improved enzymatic, chemical or electrochemical digestion efficiency. The conventional method for disulfide bond cleavage involves a time-consuming chemical reduction step using an excess of reducing agent (e.g. DTT) which has the main drawback of requiring the removal of the excess reductant prior to digestion. Van Berkel et al. were the first reporting disulfide bond cleavage within peptides by means of a PFT electrode placed at the upstream grounding point of the ESI source [66]. Free Cys residues were subsequently conjugated with oxidized hydroquinone generated at a second PFT electrode placed at the emitter tip. Chen and coworkers have further addressed and expanded the use of EC for the electrolytic cleavage of disulfide bridges using an on-

line EC-desorption electrospray ionization (EC-DESI) setup [126,127]. Glutathione disulfide (GSSG) and the three disulfide bridges of insulin were fully reduced at a potential of -1.5V vs. Ag/AgCl applied on an amalgam working electrode consisting of a thin-layer of mercury deposited onto a gold electrode surface [126]. This constitutes the first report of EC cleavage of disulfide bonds within proteins. Further investigations of biologically active peptides and proteins showed that the electrolytic cleavage of disulfide bonds provides valuable chemical information for proteomics studies [127]. The relative change in abundance of ions, that is observed when switching the EC cell on and off, can be utilized to distinguish disulfide-containing peptides from others in mixtures of enzymatic protein digests. Additionally, selective tagging of free Cys residues with selenium-based agents allows determination of the number of free thiols and facilitates the analysis and distinction of intra- and interpeptide disulfide containing peptides [128,129]. Electrolytic reduction of α-lactalbumin showed that the technique also provides conformational information. Monitoring charge state distribution shifts prior to and after electrolytic reduction revealed the role of disulfide bonds in maintaining protein conformation. Unsuccessful reduction of disulfide bridges on the contrary indicates that they are located in a buried area of the protein which hampers accessibility to the electrode surface area.

3.4.3 Further applications in peptide and protein analysis

Several other applications in peptide and protein analysis have been reported that further emphasize the potential of EC-MS in the field. For example, investigations by Girault and coworkers on the use of sacrificial electrodes for phosphopeptide tagging or for the study of biologically relevant metal ion-protein interactions expand the range of applications.

Studies of protein conformation constitute another area for EC-MS applications. As discussed in section 2.3.3 of chapter 2 EC at the liquid/metal junction of the ESI capillary can induce pH changes by oxidation or reduction of water and provoke folding or unfolding of proteins and subsequent shifts in charge state distributions [130,131]. Tertiary and quaternary structure analysis of proteins is a field where EC oxidation plays an increasingly important role. In this method, also called protein footprinting, solvent-accessible residues

are either tagged by reactive species or modified by direct oxidation. Subsequent analysis of oxidized proteins or protein complexes provides, by comparison with their unoxidized counterpart, valuable structural information. Protein surface mapping is further discussed in section 3.5 of chapter 2.

4. References

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Chapter 2

Oxidative Protein Labeling in Mass Spectrometry-Based Proteomics

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Analytical and Bioanalytical Chemistry

397, (8), 3441-3455

2010

Abstract

Oxidation of proteins and peptides is a common phenomenon, which can be employed as a labeling technique for mass spectrometry-based proteomics. Nonspecific oxidative labeling methods can modify almost any amino acid residue in a protein or only surface-exposed regions. Specific agents may label reactive functional groups in amino acids, primarily cysteine, methionine, tyrosine and tryptophan. Nonspecific radical intermediates (reactive oxygen, nitrogen or halogen species) can be produced by chemical, photochemical, electrochemical or enzymatic methods. More targeted oxidation can be achieved by chemical reagents but also by direct electrochemical oxidation, which opens the way to instrumental labeling methods. Oxidative labeling of amino acids in the context of liquid chromatography-mass spectrometry (LC-MS) based proteomics allows for differential LC separation, improved MS ionization, and label-specific fragmentation and detection. Oxidation of proteins can create new reactive groups which are useful for secondary, more conventional derivatization reactions with e.g. fluorescent labels. This review summarizes reactions of oxidizing agents with peptides and proteins, the corresponding methodologies and instrumentation, and the major, innovative applications of oxidative protein labeling described in selected literature from the last decade.

1. Introduction

The development of analytical methods for oxidatively modified amino acid residues has benefited from the study of oxidative damage to proteins related to ageing and disease [1,2]. This review focuses on applications of oxidative protein labeling in mass spectrometry-based proteomics.

Proteomics experiments typically aim at the identification and subsequent quantitation of as many proteins as possible in a sample. Often, however, a subset of proteins of interest needs to be quantified, for example, as potential biomarkers. Labeling, whether oxidative or non-oxidative, changes properties of peptides and proteins, which can be used to improve sample cleanup, HPLC separation, MS detection and quantitation. A labeling method can be tailored to enhance detection of target proteins or peptides and/or suppress signals from undesired or uninteresting ones. For example, by labeling N-terminal peptides sample complexity is greatly reduced [3], while important details about protein processing are revealed.

The main targets for derivatization in proteomics are amines (protein or peptide N-terminus and lysine (Lys)) and thiols (cysteine (Cys)) for which numerous specific labeling methods are known. Oxidative derivatization primarily targets redox sensitive residues, comprising the aromatic tryptophan (Trp), tyrosine (Tyr), phenylalanine (Phe) and histidine (His), and the sulfur-containing Cys and methionine (Met) residues (Figure 1). While thiols are usually labeled through non-oxidative electrophilic substitution reactions, disulfide bond formation between two thiol groups can be promoted by oxidizing agents and is a common and reversible labeling method.

For oxidative labeling reactions a distinction is made between primary (direct) oxidation of amino acid residues followed by reaction with nucleophiles (e.g. electrochemical oxidation of Tyr followed by reaction with water) or reaction with oxidizing reagents (e.g. hydroxyl radicals). For aromatic residues such as Tyr and Trp the distinction between oxidation and electrophilic aromatic substitution is not always clear. Different

reactions can result in the same products via different intermediates (e.g. via hydroxylation or halogenation of the aromatic rings).

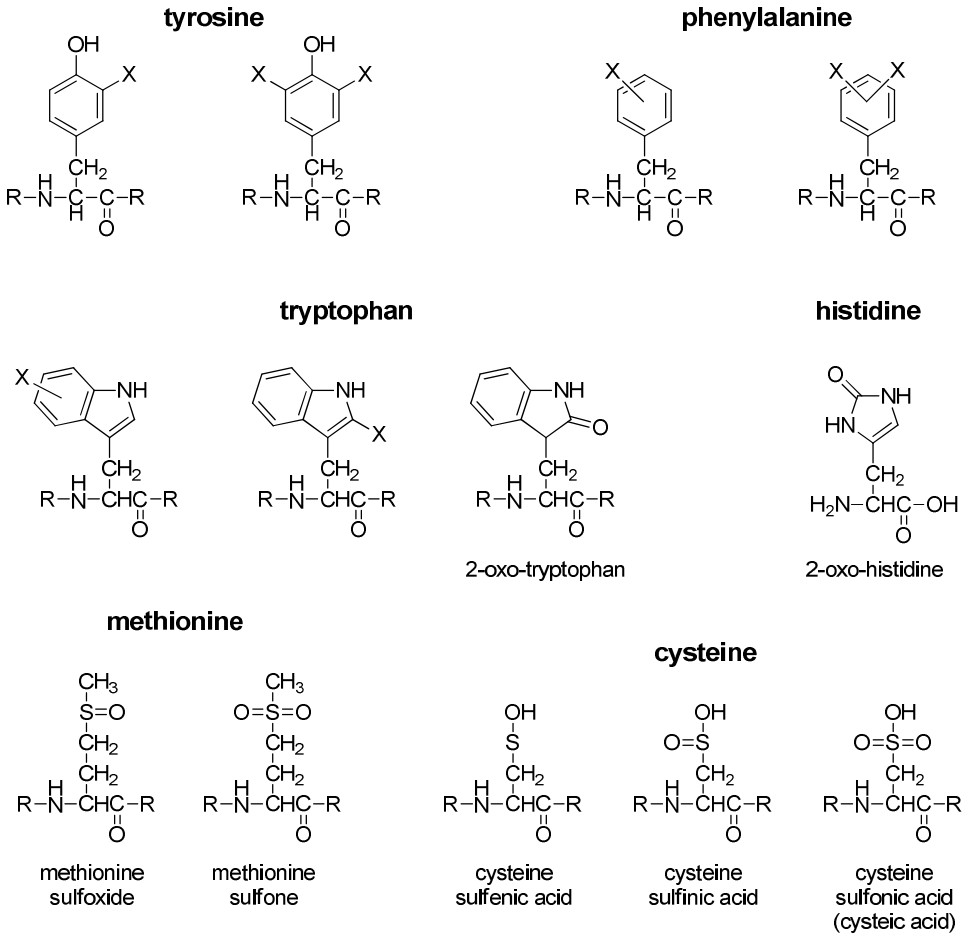

Figure 1 Structures of the most common amino acid oxidation products. X denotes either a halogen or a hydroxyl group.

Oxidative labeling is presented here as a distinct category of labeling techniques, based on the specific methods employed and the fact that less commonly targeted redox-active residues are modified. In contrast to regular chemical labeling methods, reactive intermediates of either the reactant or the target protein are formed by oxidation. Oxidative labeling is the most straightforward labeling method for aromatic residues, in particular Tyr and Trp, and provides access to purely instrumental labeling methods, such as

electrochemical (EC) oxidation, that has no equivalent in conventional, chemical labeling methods. EC oxidation can be achieved with a stand-alone electrochemical cell but the electrospray emitter itself can also act as an electrode in case of on-line (LC)-electrospray-MS analysis.

The following sections present and discuss the main reactive agents, their production methods and their most common reactions with peptides and proteins (section 2) illustrating various applications of oxidative labeling in mass spectrometry-based proteomics (section 3).

2. Methods for oxidative modification

A distinction can be made between nonspecific labeling reactions and reactions directed at specific amino acids or functional groups. The directed reactions are used for site-specific labeling while less specific methods, notably those mediated by free radicals (e.g. hydroxyl radicals), are useful for accessibility-based labeling to probe the three-dimensional structure of proteins (section 3). However, studies have shown that amino acids with redox-active side chains are more susceptible than others [4]. Nonspecific labeling reactions are also widely used for mimicking *in vivo* oxidation (e.g. susceptibility to oxidants generated during a host defense reaction).

2.1 Oxidation agents and reaction with peptides and proteins

In this section, a review of the most widely used oxidizing agents is presented and the primary end products are listed with selected examples taken from the literature. Oxidizing agents are grouped in three main categories, namely reactive oxygen (ROS), reactive nitrogen (RNS) and reactive halogen species.

2.1.1 Reactive oxygen species (ROS)

Reactive oxygen species (ROS)-mediated oxidation of amino acids in proteins is an important process occurring *in vivo* as part of the first-line defense of a host organism

against infections [5-7]. ROS-mediated protein modification has therefore been extensively studied by biologists and biochemists due to its implication in disease development [1,2]. Reactive oxygen species include hydroxyl radicals ($OH^{\bullet}$), superoxide anions ($O_2^{-\bullet}$), peroxides (ROOR, including radical species derived from them), and ozone (O_3). The effect of ROS on free amino acids and amino acid residues in proteins has been extensively studied and reviewed by Garrison [8] and more recently by Stadtman and Levine [9]. Typically, the study of ROS-mediated oxidation of proteins can be performed by generating the reactive species by chemical, photochemical, enzymatic or electrochemical reactions of molecular oxygen (O_2), hydrogen peroxide (H_2O_2) or water (H_2O).

Hydroxyl radicals are by far the most commonly studied ROS. They can easily be generated (section 2.2), and they have a broad range of reactivity, which includes not only the oxidation-sensitive Cys, Met, Trp, Tyr, Phe and His side chains, but also aliphatic groups and the peptide backbone [4,9]. Hydroxyl radicals can abstract electrons from the alpha carbon of any amino acid to form carbon radicals [4] which after reaction with O_2 lead to peptide backbone cleavage. Alternatively beta-carbon radical formation leads to beta-scission resulting in side chain cleavage [10]. Covalent modification of the side chains of aromatic amino acids by hydroxyl radicals results most commonly in hydroxylation (Figure 1). Phe is converted to 2-, 3- and 4-hydroxyphenylalanine, while Tyr yields mainly the ortho-hydroxylation product [11]. Tyr can also undergo cross-linking reactions to form dityrosine derivatives [12]. Trp residues are converted to a mixture of hydroxy-Trp isomers followed by further decomposition to kynurenine by a pyrrole ring opening reaction [13].

Ozone, as a powerful oxidizing agent, was shown to react with free amino acids and proteins similarly to other ROS by affecting sulfur-containing (Met, Cys) and aromatic residues (Trp, Tyr, Phe, His) although Phe and Cys are much less reactive. Studies with ambient air ozone [14,15] and aqueous ozone [16-19] agree on the following relative reactivity: Met > Trp > Tyr > His > Phe > Cys.

Performic acid oxidizes Met, Tyr and Trp, leading to sulfoxide formation and aromatic hydroxylation, respectively. It is used specifically for oxidative cleavage of

disulfide bonds, producing two cysteic acid residues [20,21]. Sulfenic acid is an unstable intermediate oxidation product of Cys (Figure 1). It may be further oxidized to unreactive sulfinic and sulfonic acid (cysteic acid). Sulfenic acid reacts readily with nucleophiles, including other Cys residues with which it can form a disulfide bond [22].

ROS are highly reactive and can modify most amino acid residues. As shown later in this review, such non-specific modifications are useful for protein surface mapping experiments. It is, however, of limited utility for selective, oxidative modifications.

2.1.2 Reactive nitrogen species (RNS)

Reactive nitrogen species mainly derived from nitric oxide ($NO^{\bullet}$) lead to nitration and nitrosation of proteins *in vivo* [2]. RNS, in comparison to ROS, preferentially oxidize sulfur-containing (Cys and Met) and aromatic amino acids (Tyr, Trp, Phe and His). *In vivo* nitration of Tyr is a well known widely studied phenomenon, while niration of Trp has received less attention [23]. Tyr nitration occurs at the ortho positions and dinitration is possible [24].

Peroxynitrite ($ONOO^-$), which may be formed from $NO^{\bullet}$ and superoxide anion ($O_2^{-\bullet}$), is thought to be the primary agent for reaction with Cys, Met and Trp, while for reaction with Tyr, Phe and His secondarily formed radicals are thought to be involved [24]. Reaction with NO_2 is believed to be the main *in vivo* Tyr nitration pathway [25-27].

Reaction of Cys with NO in the presence of oxygen may lead to nitrosation of the thiol group [28]. In addition, formation of sulfenic acid is possible with RNS in a similar way as with ROS [24].

RNS are more selective than ROS and thus potentially more suitable for site-directed labeling of peptides and proteins. Subsequent reduction of nitrated Tyr or Trp to their aromatic amines is a useful approach for site-directed labeling [29,30].

2.1.3 Reactive halogen species

Reactive halogen species are oxidized forms of chlorine, bromine and iodine, including hypohalous acids, which readily react with aromatic amino acids. The oxidized halogen intermediate may be formed through reaction between halide anions and oxidizers, such as H_2O_2 (section 2.2.1). Reaction with Cys and Met to form sulfoxides is possible (Figure 1) [22,31] and cystine can yield N-dichlorocystine (NDC), but halogenation of free Cys is not observed. The extent of oxidation of Cys, Met and His can be limited by using sub-stoichiometric amounts of oxidizing agents.

Reaction with Tyr and Trp leads to single or multiple halogenations (see below). Halogenated intermediates (Figure 2) react with other nucleophiles, including water, leading to hydroxylation or cleavage of adjacent peptide bonds. Oxidative halogenation and cleavage of peptide bonds C-terminal to Tyr and Trp often occur simultaneously and can be achieved by chemical or electrochemical methods (section 2.3). Some selectivity for the cleavage reaction is observed, depending on the size of the reactant and the redox activity of Tyr or Trp (at low pH the redox potential of Trp is slightly lower than that of Tyr [32]). N-Bromosuccinimide, N-Iodosuccinimide, and N-Chlorosuccinimide have been used to cleave peptide bonds C-terminal to Tyr [33-35] and Trp residues [36,37]. In all cases cleavage is accompanied by halogenation. Reagents have been proposed that are selective for Trp (e.g. *o*-Iodosobenzoic acid) but never reached widespread use [38].

Halogenation of Tyr is most favorable at the ortho position (Figures 1 and 2b) yielding a mixture of singly and doubly halogenated Tyr. For Trp the 2 position of the pyrrole ring and any position on the six-membered aromatic ring are targeted [39], analogous to naturally occurring Trp bromination patterns [40] (Figures 1 and 2a). Oxidation by reactive halogen species usually leads first to 2-oxoindole formation (Figure 2a (1)) through reaction with water. Subsequent oxidation reactions result in halogenation of the six-membered aromatic ring, presumably at the 5 position, and/or peptide bond cleavage, as illustrated in Figure 2a [39]. The secondary oxidation of the 2-oxoindole leads to intramolecular reaction to form an iminolactone, which is readily hydrolyzed, cleaving

the tryptophanyl or tyrosyl peptide bond (Figure 2a (2)). The reaction of Trp with 3 equivalents of N-bromosuccinimide (NBS) leads to monobrominated, cleaved 2-oxo-Trp [41], while the equivalent reaction with Tyr produces a dibrominated, cleaved Tyr residue [39] (Figure 2b).

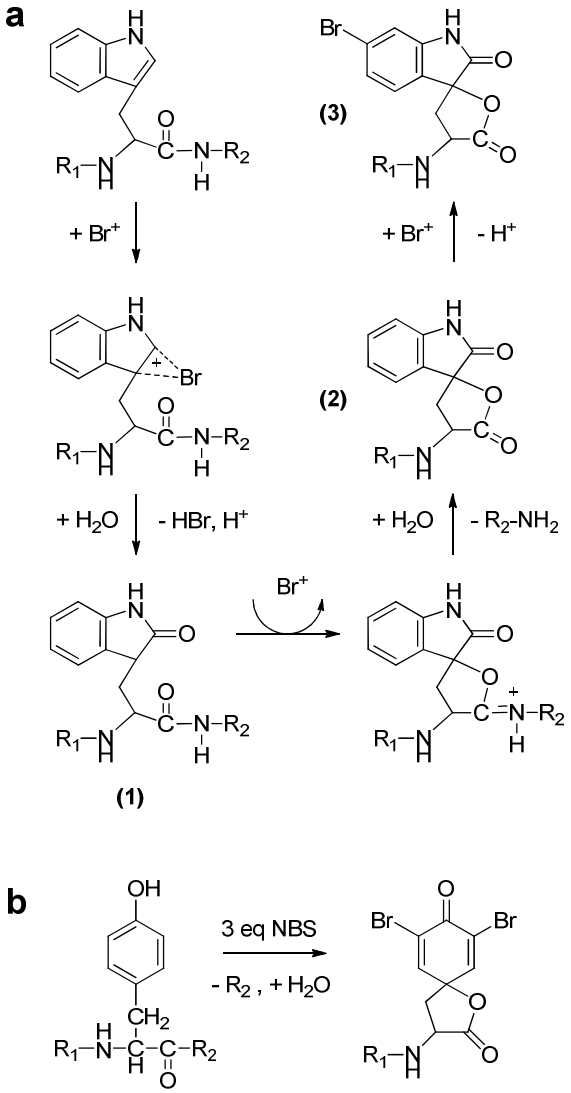

Figure 2 (a) Proposed reaction pathway for oxidation reaction of Trp with bromine [39]. A first oxidation step with Br^+ leads to the oxoindole (1), followed by a second oxidation which induces internal cleavage of the peptide bond (2). In the presence of an excess of bromine, halogenation of the six-membered ring occurs (3). (b) The analogous reaction of Tyr with 3 equivalents of N-bromosuccinimide leads to a dibrominated, cleaved Tyr residue [138].

Hypohalous acids, such as HOCl and HOBr, produce mainly halogenated Tyr and Trp, as well as 2-oxo-Trp [42-44]. In addition, many other minor modifications of these and other residues have been described [45]. Mono- and dichlorination of Tyr with performic acid in the presence of Cl^- has been observed, which may be ascribed to intermediate HOCl formation [46,47].

2.2 Production and use of oxidation agents

2.2.1 Chemical methods

Chemical oxidants can be added directly to a protein sample or generated after chemical, photochemical, electrochemical or enzymatic activation (see below). Hydrogen peroxide is the most easily obtained oxidizing agent. Hydroxyl radicals are formed from hydrogen peroxide or from water by chemical, electrochemical or photochemical activation. On-line reactions coupled to electrospray-mass spectrometry or reactions on the target plate for MALDI-mass spectrometry [48] have the advantage that sample handling is avoided.

Various peroxide species are produced by combining hydrogen peroxide and precursors, such as nitrite to form peroxynitrite [49], or formic acid to form performic acid [47]. Reactive halogen species include hypohalous acids such as HOCl and N-halogen-succinimides. Others are produced by oxidizers such as hydrogen peroxide to form hypohalous acids with molecular halogens (I_2 and Br_2) or alkali halides. Iodination of tyrosine is used in radiology, where radioactive iodine (^{125}I) is incorporated in proteins, typically with $^{125}I^-$ which is activated by chloramine T [50].

Metal catalyzed oxidation of proteins, with transition metal complexes (e.g. ruthenium bipyridine or porphyrin-like compounds) are used for photochemically or chemically-induced oxidation of Trp, Tyr and Cys to their radical intermediates [51,52]. These methods are most commonly employed for protein cross-linking or to determine the metal-binding site of proteins [53,54].

The Fenton reaction has been used to produce hydroxyl radicals from hydrogen peroxide by addition of Fe(II) for Trp and Tyr oxidation and cross-linking studies [55,56]. An Fe-complex has been constructed which produces oxoferryl (Fe(IV)=O) in combination with H_2O_2 [57], which is claimed to be potentially more specific than other ROS such as hydroxyl radical due to lower diffusion.

2.2.2 Reactive intermediates produced by irradiation

Hydroxyl radicals are photochemically generated either by photolysis of hydrogen peroxide or by radiolysis of water. Photolysis induced by a UV lamp [58] or a laser [59-65] and radiolysis induced by X-ray [66-70] and gamma-ray irradiation [71] as well as pulsed electron beam methods [72] have all proven to be useful to produce hydroxyl radicals for protein footprinting experiments (see section 3.5).

Photochemical oxidation of hydroquinone with a UV laser on a MALDI target coated with TiO_2 particles has been shown to produce benzoquinone, that reacts with thiols in peptides [73]. This parallels the electrochemical generation of benzoquinone in the electrospray emitter reported by the same group (section 2.3.2). Furthermore, metal complexes employed in protein oxidation can be photochemically activated, e.g. the ruthenium-bipyridine complex [52].

2.2.3 Electrochemically produced reactive intermediates

Electrochemistry is a useful method of producing ROS in a controlled manner. An electrochemical cell can be used to oxidize water to hydroxyl radicals at sufficiently high potential. Boron-Doped Diamond (BDD) electrodes are particularly suitable for hydroxyl radical production due to their intrinsic high overpotential for the formation of molecular oxygen by oxidation of water (oxygen evolution) [74,75]. The potential at which the radical formation is observed is dependent on the doping and impurities level of the BDD material employed [75]. Another major advantage of BDD when it comes to protein analysis is its low adsorption. Adsorption of proteins and peptides to the working electrode affects reproducibility as has been observed when working with commonly used carbon electrodes [76]. Hydroxyl radical formation at high voltage combined with the low adsorption

properties makes BDD a suitable and efficient material for footprinting experiments ([77]; see section 3.5).

The Fenton reaction (section 2.2.1) can be initiated in an electrochemical cell [78]. Alternatively, the electrospray emitter of a mass spectrometer's source can be employed to oxidize atmospheric oxygen. High voltage on the electrospray emitter tip (typically above 5 kV) can generate a corona discharge, which leads to hydroxyl and perhydroxyl radical formation and subsequent peptide oxidation in the gas phase [79], an approach that has been used for protein footprinting experiments [80,81] (see section 3.5). However, Boys *et al.* showed recently that corona discharge can occur under regular electrospray ionization (ESI) conditions (3.5 kV, N_2 nebulizer gas) inducing hemoglobin oxidation [82]. Electrochemical production of RNS in an electrochemical cell has been shown to lead to tyrosine nitration [83,84] by oxidation of nitrite to nitrogen dioxide or peroxynitrite.

2.2.4 Enzymatically produced reactive intermediates

Peroxidases are often used for production of ROS or RNS in *in vivo* assays. Both the enzyme and an oxidizing reagent may be added to a protein sample, but more commonly peroxidases are supplied with hydrogen peroxide to induce *in situ* oxidation. For example, myeloperoxidases can produce HOCl from Cl^- and H_2O_2, and NO_2 from nitrite and H_2O_2 [85], leading to Tyr chlorination or nitration, respectively.

The tyramine labeling system [86] uses peroxidase activity to cross-link tyramine to Tyr residues in proteins. Tyramine-fluorophore labelling has been used to probe Tyr radical formation *in vivo* [87].

2.3 Direct electrochemical oxidation of peptides and proteins

2.3.1 Electrochemical cell

Electrochemistry has long been a domain reserved to specialists but the coupling of electrochemistry to mass spectrometry has generated increased interest due to its ability to monitor electron transfer processes on-line. Many groups have contributed to the

development of methods and instrumentation for coupling of electrochemical cells to MS and LC-MS systems (see [88-90] for reviews).

Electrodes can donate or accept electrons and thus reduce or oxidize macromolecules. Metalloproteins have for instance been widely studied because of their crucial role in *in vivo* redox processes [91]. Electrochemistry of biomacromolecules is mainly used for detection purposes and in that aspect metalloproteins are easily oxidizable targets due to their metal ion cofactor.

Electrochemistry is also used to mimic oxidative modifications occurring *in vivo* through enzymatic and ROS activity. Indeed, proteins contain electroactive amino acid residues which side-chains can interact with an electrode and undergo electron transfer reactions. Sulfur-containing Cys and Met residues as well as the aromatic Tyr, Trp and His are susceptible to direct oxidation reactions in an electrochemical cell when positive potential is applied to the working electrode. Reduction of disulfide bridges can, on the other hand, be achieved by applying negative potentials [92].

Electrochemical oxidation of aromatic amino acids produces reactive intermediates which mainly yield hydroxylated residues by reaction with water and, for Tyr, quinones after secondary oxidation reactions. Sulfur-containing residues can be oxidized through up to three oxygen atom insertions in the case of Cys [90]. Another common oxidation reaction for Cys is to form a disulfide bond with another Cys residue. Recent work in our group showed that cleavage of the protein backbone can occur after electrochemical oxidation of peptides and proteins [76,93]. Similarly to the chemical cleavage with halogen species described above (section 2.1.3), electrochemical oxidation of peptides and proteins leads to the preferred cleavage of peptide bonds C-terminal to Tyr and Trp residues. The cleavage reaction yields a spirolactone derivative of Tyr and Trp (Figure 2). This amino acid specificity makes it a promising method for the development of an alternative protein digestion technique. Interestingly it has been shown that the method can be used for distinguishing phosphorylated Tyr from Tyr, since phospho-Tyr cannot be oxidized and thus no cleavage occurs [93].

Proteins have not been extensively studied in terms of oxidative side-chain modifications using electrochemistry although it is a potentially useful method to mimic *in vivo* processes. Brabec *et al.* [94,95] first presented electrochemical oxidation of Tyr and Trp side chains in the protein backbone. Nitration of Tyr side-chains in lysozyme has also been investigated by electrochemical oxidation of nitrite [84].

Carbon-based working electrodes are the most commonly used in organic electrochemistry but present the main drawback of surface oxidation and fouling due to irreversible adsorption when working with large peptides and proteins. This strongly hampers accurate analysis and reproducibility. Boron-doped diamond (BDD) electrodes have recently gained considerable attention due to their chemical inertness, large potential window and more importantly their lower adsorption properties as compared to glassy carbon [74,75]. Shin *et al.* showed strongly reduced adsorption of Bovine Serum Albumin (BSA) to BDD electrodes as compared to glassy carbon [96] and Chiku *et al.* investigated the direct electrochemical oxidation [97] and conformational changes of BSA [98] using BDD electrodes indicating that this electrode material is suitable for protein analysis.

2.3.2 Electrospray emitter

The simplest configuration to perform electrochemical oxidation in combination with mass spectrometry is to make use of the inherent electrochemical processes occurring within the electrospray emitter of the mass spectrometer. Kebarle and coworkers were the first to compare the ESI source to an electrolytic cell by showing that Zn(II) and Fe(II) ions were generated in solution by electrochemical oxidation of the emitter tip [99]. Electrospray ionization by its nature involves electrochemical processes. In positive ionization mode, positively charged droplets formed at the emitter tip are flowing towards the aperture of the mass spectrometer due to the applied electrical field. The way to balance charges and to allow current to flow through the entire circuit is by providing electrons at the liquid-metal interface by electrochemical oxidation of the metallic emitter tip and/or electrolytes present in solution to release electrons although some discussion is still ongoing about this process [100,101]. The emitter can thus act as a working electrode where oxidation (positive ion

mode) or reduction reactions (negative ion mode) are taking place whereas the curtain plate or inlet capillary of the mass spectrometer plays the role of the counter electrode. Van Berkel and coworkers studied the fundamental aspects of the electrospray ionization process extensively [101] and "in source" redox reactions were mainly used to ionize compounds such as porphyrins [102], polyaromatic hydrocarbons [103], metallocenes [104], fullerenes [105] in the form of radical cations in positive ion mode.

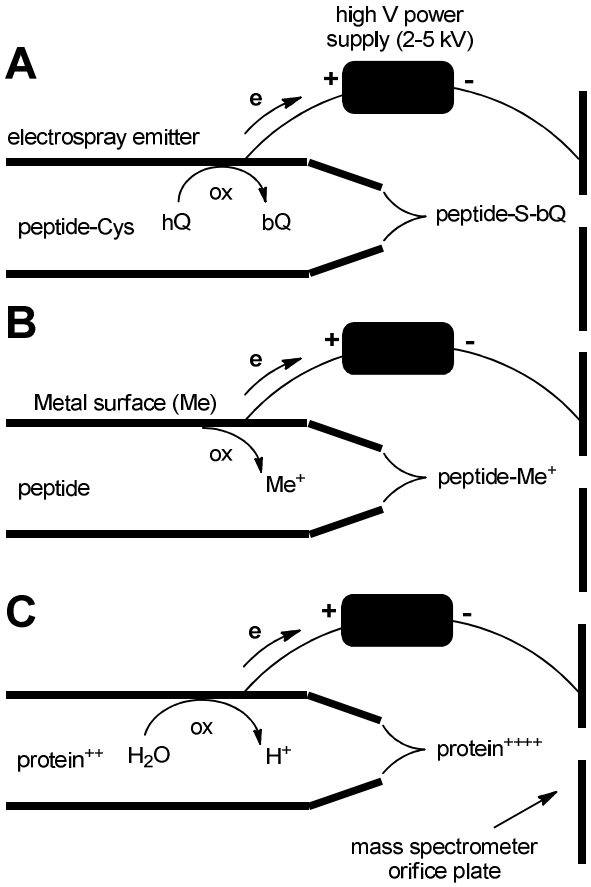

Figure 3 Oxidation reactions in an electrospray emitter. (a) Oxidation of hydroquinone (hQ) to benzoquinone (bQ) in the presence of Cys-containing peptides leads to quinone-labeled peptides [106]. (b) Oxidation of metals (e.g. from the emitter material itself) leads to metal ions that may coordinate with peptides [113]. (c) Oxidation of water reduces the solvent pH, leading to higher protein charge states [121].

Girault and coworkers pioneered the use of in source redox reactions for peptide and protein analysis. In their work, hydroquinone was oxidized to benzoquinone that reacts with the thiol groups of Cys in peptides and proteins within the emitter as shown in Figure 3A [106-110]. In this way they developed a system for 'on-line counting' of Cys residues with the goal to facilitate peptide identification through database searches, which is enhanced when at least one amino acid residue is known in the sequence [109,111,112]. The technique was shown to be successful on a set of peptides containing up to three Cys residues and on proteins, although tagging efficiency decreased with increasing molecular size due to steric hindrance and slower kinetics.

The same group explored the use of sacrificial electrodes on microchip devices to generate metal ions in solution (Cu(II), Zn(II), Ni(II), Fe(II), Ag(I)) that can complex to peptides (Figure 3B) [113]. The use of copper and zinc electrodes appeared to be most efficient to probe metal ion-protein interactions that mimic those observed in biological systems. Copper sacrificial electrodes for instance were used to study copper-Cys interactions [114-116]. Copper is known to catalyze Cys oxidation *in vivo* [117] and it was shown that generating Cu(I) and Cu(II) ions in solution promoted the formation of inter- or intra-molecular disulfide bridging. Alternatively a sacrificial zinc electrode was used to tag phosphopeptides. Zn(II) ions generated at the emitter tip were shown to interact with phosphopeptides and complex phosphorylated Tyr and Ser [116,118].

Redox reactions taking place at the electrospray emitter electrode can affect the composition of the solution in the capillary [119,120]. Oxidation or reduction of water at the liquid-metal interface (Figure 3C) can induce variation of the pH in solution. McLuckey and coworkers [121] showed that this change of solution composition can be used to enhance ubiquitin, myoglobin and cytochrome c detection in negative ion mode. Indeed reduction of water within the electrospray capillary progressively increased the basicity of the solution, through formation of OH^- ions, thus altering the charge state of the different proteins and improving the detection in negative ESI-MS. The electrochemical modification of a solute inside the electrospray capillary is strongly dependent on liquid flow rate. When the flow rate is increased there is a lower yield of electrochemical reaction products [101].

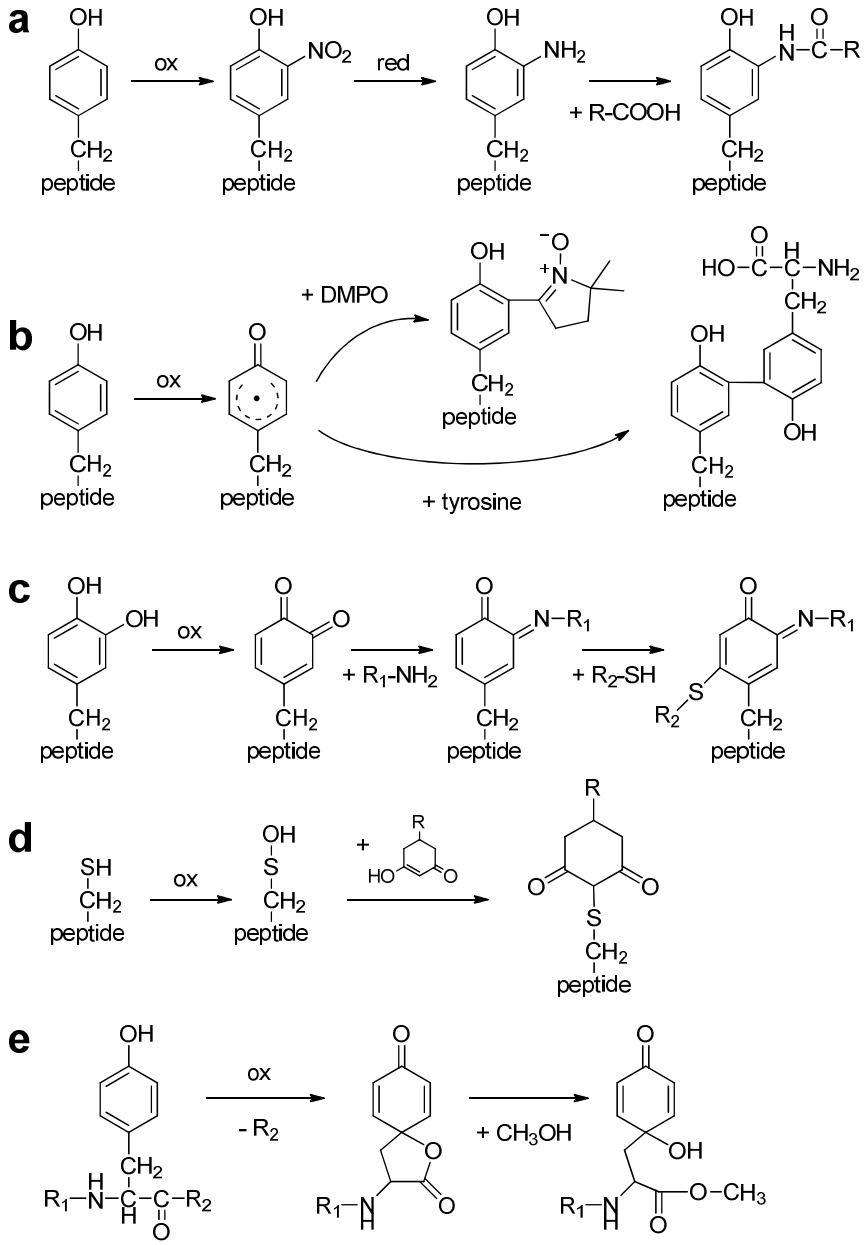

Figure 4 Secondary labeling reactions of oxidized amino acid residues. (a) Oxidative nitration, followed by reduction of the nitro-group to an amine, and finally amide formation [27,131] (b) Reaction of the radical intermediate of Tyr with the spin-label DMPO [134] or a second Tyr residue [51] (c) Secondary oxidation of hydroxylated Tyr to an orthoquinone, followed by reaction with an amine (e.g. a protein Lys), leading to a Schiff base intermediate, which is very reactive towards thiols [51] (d) Reaction of sulfenic acid with a linker molecule containing secondary amine or azide functional groups for labeling [137] (e) Tyr peptide bond cleavage leads to a lactone intermediate that can react with alcohols or amines [138].

3. Applications

Applications of chemical modifications and tagging strategies for protein research and proteomics are covered in recent reviews [122-124]. Here we highlight applications where oxidation facilitates sample preparation and purification, and those where oxidation changes physico-chemical properties with respect to chromatographic separation, ionization efficiency or mass are utilized for improved or differential detection of labeled peptides.

3.1 Sample preparation

Oxidative modification for sample preparation is most commonly performed at the protein level. The oxidized functional group may be the end point of labeling, but oxidation methods can also be employed for creation of functional groups for secondary labeling, e.g. biotinylation for affinity purification or fluorescent labeling for detection.

Figure 4 illustrates a variety of secondary labeling methods after oxidation of amino acids. Nitro-Tyr can be reduced with sodium disulfite [125], dithiothreitol (DTT) in combination with heme [126] or by electrochemical reduction [127-130] and yield amino-Tyr. This amine group is useful for coupling labels (Figure 4a) such as biotin [27,30,131], sulfhydryl groups [29] or fluorescent tags [132]. This strategy allows for isolating residues containing the modified amino acids. Zhang et al. and Abello et al. show selective tagging of Nitro-Tyr that is achieved after prior acetylation of primary amines [29,30].

Oxidative cross-linking of radical intermediates of Tyr with other Tyr residues (Figure 4b) or with tyramine coupled to a fluorophore [51,52,87] produces dityrosine-containing compounds with distinct spectroscopic properties. The study of cross-linking reactions are of great interest due to the fact that they involve residues (Tyr, Trp, Cys) that often play an essential role in protein function and biological recognition processes. Such studies have for instance revealed intermolecular interactions between G-protein-coupled receptors (GPCRs) within intact cells [51] or that neutrophil-derived proteins with anti-inflammatory and bactericidal properties are more susceptible to be attacked by Tyr radicals [87]. The biological role of protein Tyr radicals can be assessed by trapping with the spin-

label 5,5-dimethylpyrroline-N-oxide (DMPO) before reacting with other radicals (Figure 4b). The trapped radicals are stable enough for subsequent tryptic digestion and MS analysis and can thus be localized in the protein [133,134]. Those studies allow to map the radicals in proteins under oxidative stress leading to a better understanding of the mechanism that are related to human diseases. Trapped Tyr residues can also be detected with antibodies and by molecular magnetic resonance imaging (mMRI) [135].

Tyr ortho-quinone is produced by a second oxidation after oxidative hydroxylation (section 2.3.1). This group is susceptible to nucleophilic attack by thiols, as shown in Figure 4c, or by other oxidized Tyr residues [51,136]. Sulfenic acid is capable of nucleophilic attack which has been used to couple a linker molecule that subsequently reacts with fluorescent labels [137] (Figure 4d).

The spirolactone group of Tyr, which is produced upon peptide cleavage, is reactive towards alcohols and amines under basic conditions to form esters and amides (Figure 4e) [138]. An analogous reaction with amines such as tris(hydroxymethyl)aminomethane has been described for the homoserine lactone produced by CNBr cleavage C-terminal to Met [139].

Protein digestion for proteomics, which is typically performed enzymatically, may be achieved by electrochemical or chemical oxidative cleavage of proteins as described in sections 2.1.3 and 2.3.1. The distinct specificity and the reactive spirolactones of Tyr and Trp at the peptide C-terminus may provide novel peptide enrichment and identification strategies.

3.2 LC properties

Oxidative labeling may change peptide retention on chromatographic stationary phases. Hydroxylation of Tyr and Trp and oxidation of Met and Cys to sulfoxide or sulfo-acids decreases their hydrophobicity thus reducing peptide retention on reversed phase (RP) columns. Hydrophobicity and elution order may be further affected by solvent composition

and pH (e.g. nitro-Tyr has a longer retention time than Tyr on RP-HPLC under acidic conditions, but elutes earlier under basic conditions [27]). Increased retention times are observed upon halogenation, so that Tyr, bromo-Tyr and dibromo-Tyr elute in this order in RP-HPLC [140].

Direct comparison of labeled versus unlabeled samples is possible, but as for peptide mass shifts (section 3.3.2), sample complexity and low abundance of peptides of interest can make retention time shifts hard to detect. To overcome this issue the COFRADIC method has been developed [3], which is a two-stage LC method with a primary separation, labeling of certain molecules or functional groups in fractions, and a secondary separation. Differential labeling will cause a shift in retention time for labeled peptides while unlabeled peptides remain unaffected, making labeled peptides easily detectable. COFRADIC has been applied to various targets based on different labeling methods, including oxidation of Met with performic acid [141]. Met oxidation has also been used to reduce the hydrophobicity of peptides derived from membrane proteins, which are difficult to elute from RP stationary phases [142]. This is a case where oxidative modification of methionine residues, that are often located in transmembrane segments, leads to increased peptide coverage, which is often an issue when analyzing membrane proteins by LC-MS. Reaction of Cys with benzoquinone, similar to the electrospray tagging reaction (section 2.3.2), has been employed to induce a hydrophilic shift in COFRADIC with RP-HPLC [143].

3.3 Mass spectrometric properties

3.3.1 Ionization efficiency and charge state

Protein solution-phase conformation influences the charge state of a protein during the ESI process [144,145]. The charge state itself influences the ionization efficiency of proteins since highly charged conformers are more easily amenable to ESI. A protein in its native folded conformation gives rise to lower charge states (fewer available protonation sites) and a wider charge distribution compared to its unfolded equivalent. Based on this empirical relationship, ESI-MS has been used as a tool for monitoring protein conformational changes in solution [146] including for protein footprinting experiments

[147] (section 3.5) where oxidative modifications affect the solution-phase structure. The nature of the oxidized residue can also affect the charge state as testified for instance by the lower proton affinity of 2-oxo-His compared to unmodified His [148]. Oxidation of Cys to cysteic acid can on the other hand contribute to an increase of charge state in negative ion mode due to its acidity. It is also noteworthy that the peptide dissociation patterns in MS/MS may be affected by oxidatively modified residues, which must be taken into account during the interpretation of MS/MS spectra [148] (section 3.3.2). Ionization efficiency can also be increased by taking advantage of inherent electrochemical processes taking place in the electrospray emitter as discussed in section 2.3.2.

3.3.2 Mass change of peptides and fragments

Almost any labeling method, including oxidation, will increase the mass of an analyte. Differential MS analysis of oxidized versus unoxidized samples will therefore reveal modified peptides, provided that they are sufficiently abundant and can be ionized well. Alternatively, much more sensitive MS/MS methods can selectively detect modified peptides based on characteristic product ions or neutral losses [149]. Table 1 lists precursor and neutral loss scanning applications that have been described for various (*in vivo*) modifications that can be mimicked by oxidative labeling.

Collision-induced dissociation (CID) of peptides is influenced by side chain properties of the constituent amino acids. Notably, creation of acidic or basic groups affects the proton localization, so that formation of sulfinic and sulfonic acid leads to greatly increased fragmentation at their C-terminal side [150], analogous to the preferential fragmentation induced by aspartic acid [151]. This observation may help in interpreting MS/MS spectra of unknown peptides [152]. In contrast, the preferential fragmentation normally observed for His is inhibited upon its oxidation to 2-oxo-His [148].

While fragmentation of protonated peptides by CID is the most common technique in proteomics, electron transfer-induced dissociation (ETD) has gained considerable interest, since its introduction in 2004 [153], and applications targeting oxidized residues are

easily envisaged. In a related method, iodinated Tyr is irradiated in the gas phase to produce radical intermediates which readily fragment upon collisional activation [154].

Residue	Modification	Precursor immonium ion (Da)	Neutral loss (Da)	Reference
Trp	bromo	237.002 239.001[a]		[155]
	hydroxy	175.086		[45]
Tyr	hydroxy	152.071		[45]
	bromo	213.986		[174]
	chloro	170.037		[45,174]
	nitro	181.061		[175]
	dinitro	226.046		
	iodo	261.973		[176]
	diiodo	387.869		
Met	sulfoxide		63.998	[177,178]
Cys	sulfinic acid		65.977[b]	[150]
	cam-sulfoxide[c]		107.004	[179,180]
	cam-sulfone[c]		122.999	

Table 1 Specific fragments of oxidatively modified amino acid residues used for MS/MS based detection. Notes: [a] ^{81}Br isotope; [b] negative ionization; [c] cam denotes carbamidomethylcysteine produced by alkylation with iodoacetamide.

3.3.3 Mass defect and isotopic pattern signature

In addition to the overall mass increase upon labeling, the elemental composition of the labeling group may introduce a shift in the mass defect of the peptide or protein, which is on average +0.00055 amu per amu for protein-derived peptides. Labels with many hydrogen atoms cause an additional positive mass defect, while incorporation of heavier elements, including chlorine, bromine and iodine, lead to an increasingly large negative mass defect (Table 2). The term Mass-Deficient Mass Tag (MaDMaT) has been proposed by Steen and Mann [155]. The large mass defect of halogens may be used for the preparation of internal standards for quantitative proteomics [156]. MaDMaT-labeled peptides are easily distinguished from non-labeled peptides in a mass spectrometer with sufficient mass resolution and accuracy [156]. Performic acid oxidation of Met [20], or

halogen labeling [157,158] expand the mass distribution of tryptic peptides and the compositional information may be used to improve peptide identification.

The well-known specific isotope patterns of chlorine and bromine are used to confirm their presence and number from the relative isotope intensities (Table 2). In combination with its large mass defect, the characteristic isotope pattern makes detection of labeled peptides in mass spectra rather straightforward.

3.4 Differential isotopic labeling for quantitation

Isotopic labeling is a standard approach in mass spectrometric quantitation, and becoming increasingly important in proteomics studies. Synthesis of labeled peptides as well as metabolic and enzymatic labeling is widely used, but the most common method is chemical labeling either at the peptide or protein level. The primary labeling targets are the same as for non-isotopic labeling, such as primary amines of Lys residues or protein/peptide N-termini [159,160] or the thiol of Cys [161]. Labeling is performed by reaction with alkyl-halide (nucleophilic substitution) or maleimide (thiols), or N-hydroxysuccinimidyl esters (amines). Trp is also a target for direct electrophilic aromatic substitution using the sulfenylation reagent 2-nitrobenzenesulfenyl chloride [162].

Oxidative labeling for quantitation is limited by the lack of specificity and completeness of the reactions, but it is certainly useful if incomplete labeling is acceptable. Other applications described to date comprise oxidative tagging with $H_2{}^{18}O$ during electrospray ionization [163] and metabolic isotopic labeling of amino acid targets followed by H_2O_2 oxidation, as shown for Met in the COFRADIC workflow [164].

Modifi-cation	chemical composition	monoisotopic mass ($M+H^+$, Da) and mass defect	average tryptic peptide monoisotopic mass ($M+H^+$, Da)	lowest tryptic peptide monoisotopic mass ($M+H^+$, Da)	isotope masses and relative intensities
none	$C_{62}H_{90}N_{17}O_{14}$	1296.6848 (0.52 mmu)	1296.6475 (- 0.0373 Da)	1296.5526 (- 0.1322 Da)	1296.68 100.0 1297.69 74.9 1298.69 30.6 1299.69 8.9 1300.70 2.0
hydroxy	$C_{62}H_{90}N_{17}O_{15}$	1312.6797 (0.52 mmu)	1312.6706 (- 0.0091 Da)	1312.5286 (- 0.1511 Da)	1312.68 100.0 1313.68 74.9 1314.69 30.8 1315.69 9.1 1316.69 2.1
chloro	$C_{62}H_{89}N_{17}O_{14}Cl$	1330.6458 (0.49 mmu)	1330.6723 (+ 0.0265 Da)	1330.5344 (- 0.1114 Da)	1330.65 100.0 1331.65 74.9 1332.65 62.5 1333.65 32.8 1334.65 11.8 1335.65 3.2
bromo	$C_{62}H_{89}N_{17}O_{14}Br$	1374.5953 (0.43 mmu)	1374.6884 (+ 0.0931 Da)	1374.5483 (- 0.0470 Da)	1374.60 78.2 1375.60 58.6 1376.60 100.0 1377.60 63.9 1378.60 24.9 1379.60 7.1 1380.60 1.6
iodo	$C_{62}H_{89}N_{17}O_{14}I$	1422.5814 (0.41 mmu)	1422.7089 (+ 0.1275 Da)	1422.5259 (- 0.0555 Da)	1422.58 100.0 1423.58 74.9 1424.59 30.6 1425.59 8.9 1426.59 2.0
dibromo	$C_{62}H_{88}N_{17}O_{14}Br_2$	1452.5058 (0.35 mmu)	1452.7232 (+ 0.2174 Da)	1452.5795 (+ 0.0701 Da)	1452.51 44.4 1453.51 33.3 1454.50 100.0 1455.51 68.7 1456.50 69.3 1457.51 39.3 1458.51 14.6 1459.51 4.1

Table 2 Effect of oxidative modifications of Tyr on the mass defect and isotope pattern of angiotensin I (DRVYIHPFHL). The average and lowest monoisotopic masses of tryptic peptides (without missed cleavages) were calculated in 1 Da mass bins from a yeast protein database (www.yeastgenome.org; orf_trans.fasta database of 8 May 2009).

3.5 Protein surface mapping

Protein 'footprinting' is a technique that gained attention during the last decade due to its ability to probe solvent accessible residues. The technique of choice makes use of *in situ* generated hydroxyl radicals that induce oxidative chemical modification of surface accessible reactive amino acid side-chains. The term footprinting was introduced some 30 years ago by Galas *et al.*, who performed DNA surface mapping [165]. H/D exchange experiments are also used to probe surface accessible residues by mass spectrometry but the need to quench the reaction at low pH to avoid back-exchange reactions during protein analysis limits its usefulness, since only a few proteases (e.g. pepsin) work under low pH conditions (see [147] and [166] for reviews). We will focus here on the use of hydroxyl radicals that allowed considerable improvement in terms of spatial resolution compared to bulky proteases used in other methods [167-169]. In addition, the fact that the small hydroxyl radicals nonspecifically attack a wide range of residues makes them better suited to probe solvent accessibility and thus a protein's tertiary and quaternary structure. Sulfur containing (Cys, Met, cystine) and aromatic residues (Trp, Tyr, Phe) represent the most reactive targets for oxidative covalent modification but other side-chains (His, Leu, Ile, Arg, Lys, Val, Pro, Gln, and Glu) have also been shown to be modified [66-68,70,77,170,171]. Details about the chemical reactions underlying the covalent modification of amino acid side chains and backbone cleavage can be found in a detailed review by Xu and Chance [4].

The general footprinting workflow (Figure 5) consists of producing hydroxyl radicals which can be achieved by several means (see below and section 2.2) and different exposure times. After exposure, proteins are digested with proteases and the extent of oxidation is measured by MS. The location of oxidized amino acids is finally assigned by MS/MS analysis. The obtained results provide information about the solvent-exposed residues on the surface of the protein and can also lead to the determination of protein-ligand interaction sites by comparison of protein and protein/ligand complexes. When protein oxidation is measured as a function of exposure time to the hydroxyl radicals, conformational changes can be followed due to increased amino acid accessibility by protein unfolding induced by primary oxidation events.

A wide set of techniques to produce hydroxyl radicals has expanded the toolbox available for footprinting experiments (see section 2.2). The Fenton reaction to produce hydroxyl radicals chemically [55,56] has the main drawback that iron and EDTA may distort the native protein conformation. Photochemical formation of hydroxyl radicals by photolysis of hydrogen peroxide or by radiolysis of water are very efficient and provide the advantage (compared to the Fenton reaction) of fast (nano- to microseconds) radical generation, and the possibility of short exposure times (less oxidation-induced structural changes) allowing time-resolved studies. Radiolysis has the advantage of not requiring the addition of any reagent to the sample solution. Electrochemistry may also be used to generate reactive oxidants for mapping solvent accessibility. Whereas Brabec *et al.* have pioneered the use of EC to probe protein conformational changes [95,172], new approaches for EC-based probing of the higher-order structure of proteins have been introduced by McClintock *et al.* [77] where hydroxyl radicals have been produced by oxidation of water on a boron-doped diamond electrode. Oxidation in the electrospray source by corona discharge has also been performed [81,173].

Protein footprinting experiments have found widespread application to probe protein structure and solvent accessible residues in the native conformation. Moreover probing protein-protein, protein-ligand and protein-drug interaction sites as well as monitoring conformational changes (e.g. protein folding and unfolding) have been studied extensively, as shown by the many applications that have been reviewed in recent years [4,68,80,147,171]. Although footprinting methods have matured, some drawbacks such as secondary oxidation reactions are still an issue. It was observed that buried sulfur-containing residues can be oxidized either by internal electron transfer reactions [58] or by secondary oxidations due to residual hydrogen peroxide or peptidyl hydroperoxides [65]. Oxidation of non-solvent accessible residues can hamper data interpretation and must be considered when performing footprinting experiments. Adding catalase and/or Met as scavengers limits secondary oxidations that may occur particularly on (buried) sulfur-containing residues [65] but internal electron transfer reactions are still difficult to avoid.

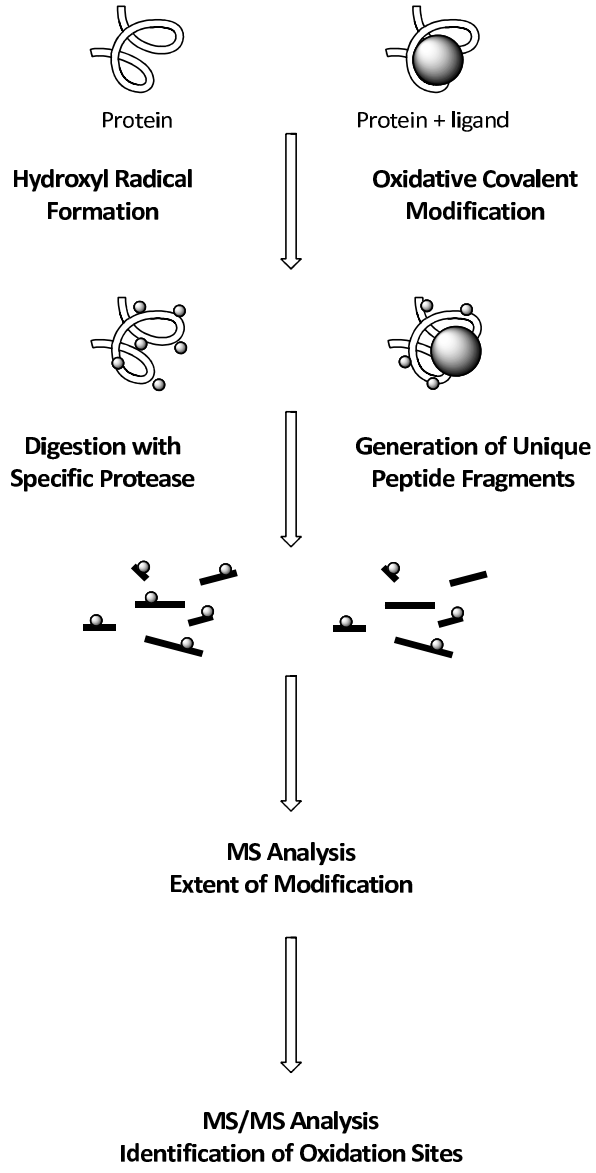

Figure 5 Workflow for hydroxyl radical footprinting of proteins and protein/ligand complexes (adapted from Xu and Chance [4]).

4. Conclusion

Oxidative labeling of peptides and proteins has expanded the toolbox of biochemists and notable progress has been made in the past decade. A variety of strategies for targeting amino acid side-chains has been developed, in particular for sulfur-containing and aromatic residues. Current labeling techniques targeting primary amines (N-termini, Lys) and cysteine residues have found widespread applications in proteomics while other amino acids remain inaccessible to labeling to a large extend. The increased interest in oxidative labeling stems from the possibility to target other amino acid residues, such as Tyr, Trp or Met, which have essential roles in protein function and biological recognition processes. Although the technique still suffers in many cases from incomplete conversion and selectivity, several applications in biological systems have shown its usefulness. It is, for instance, to be expected that oxidative labeling will help to improve our understanding of protein-protein interactions with respect to protein function and signaling processes.

An interesting feature of oxidative labeling is its potential to introduce reactive groups in a site-specific manner, which can be targeted by secondary chemical reactions as shown for nitro-Tyr residues. Electrochemistry, which is far from being fully exploited, can efficiently induce such oxidative modifications. Many advances and developments have been observed in this field during the last decade and interesting applications can be envisaged especially due to new developments in electrode materials and surface-modified electrodes. Boron-doped diamond electrodes, for example, are of interest due to their reduced adsorption of biomolecules opening the way for new application of direct and ROS-mediated oxidation of peptides and proteins.

The increasing demand for mass spectrometry-based proteomics methods and the relationship with *in vivo* oxidative protein damage through radical-induced modifications suggest that these techniques will find increasing use.

5. References

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Chapter 3

Electrochemical Oxidation and Cleavage of Tyrosine- and Tryptophan-containing Tripeptides

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Analytical Chemistry

82, (18), 7556-7565

2010

Abstract

Electrochemical oxidation of peptides and proteins has been shown to lead to specific cleavage next to tyrosine (Tyr) and tryptophan (Trp) residues which makes the coupling of electrochemistry to mass spectrometry (EC-MS) a potential instrumental alternative to chemical and enzymatic cleavage. A set of Tyr and Trp-containing tripeptides has been studied to investigate the mechanistic aspects of electrochemical oxidation and the subsequent chemical reactions including peptide bond cleavage, making this the first detailed study of the electrochemistry of Trp-containing peptides. The effect of adjacent amino acids was studied leading to the conclusion that the ratios of oxidation and cleavage products are peptide-dependent and that the adjacent amino acid can influence the secondary chemical reactions occurring after the initial oxidation step. The effect of parameters such as potential and solvent conditions showed that control of the oxidation potential is crucial to avoid dimer formation for Tyr and an increasing number of oxygen insertions (hydroxylations) for Trp, which occur above 1000 mV (vs. Pd/H_2). While the formation of reactive intermediates after the first oxidation step is not strongly dependent on experimental conditions, an acidic pH is required for good cleavage yields. Working under strongly acidic conditions (pH 1.9 to 3.1) led to optimal cleavage yields (40-80%) whereas no or little cleavage occurred under basic conditions. On-line EC-MS allowed determining the optimal potential for maximum cleavage yields, whereas EC-LC-MS/MS revealed the nature and distribution of the reaction products.

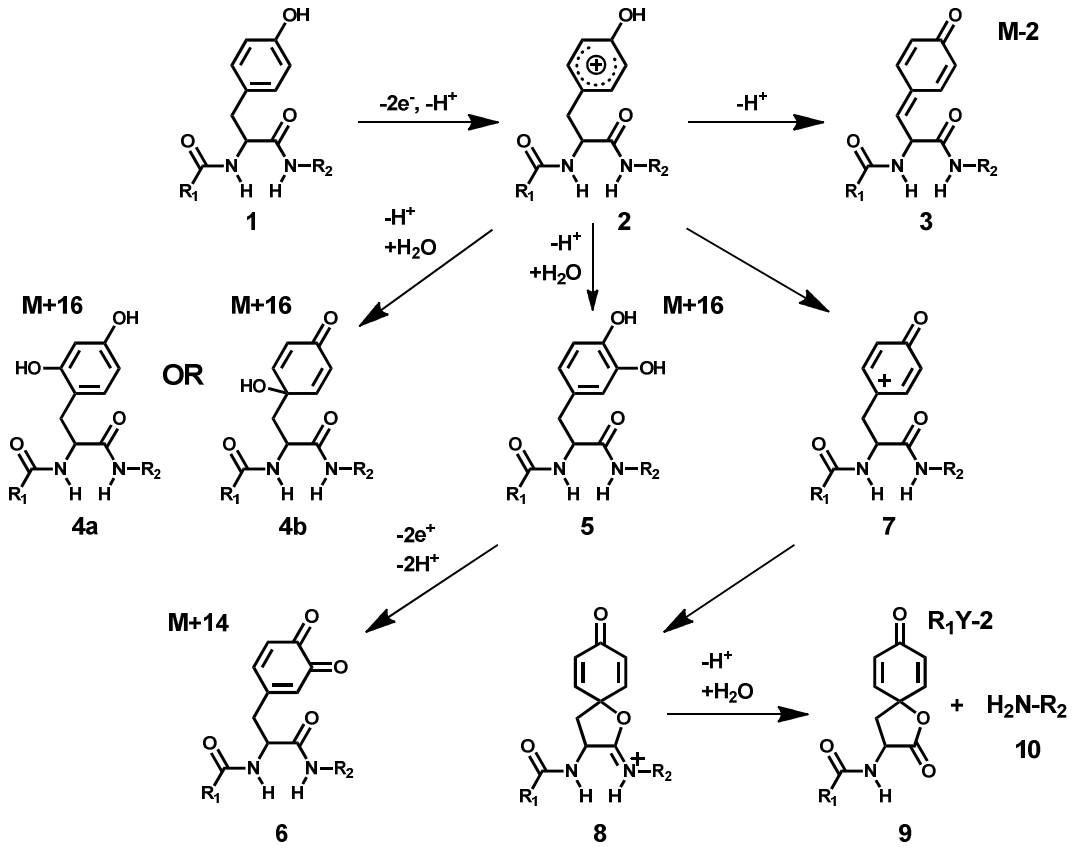
1. Introduction

Oxidation is a major modification of peptides and proteins that is related to disease and ageing processes. Proteins are oxidized *in vivo* and *in vitro* through chemical, photochemical, electrochemical or enzymatic reactions generating reactive intermediates, such as hydroxyl radicals or peroxides. Depending on the reactive intermediate, any amino acid can be oxidized, although the most susceptible residues are the sulfur-containing cysteine (Cys) and methionine (Met) and the aromatic phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp) and histidine (His) [1-3]. A unique feature of electrochemical oxidation is the ability to directly abstract electrons from functional groups to the electrode. Cys, Met, His, Tyr and Trp are susceptible to direct oxidation and electrochemical detection is used in protein and peptide analysis [4]. Recently, electrochemical protein oxidation has been used for oxidative footprinting to probe solvent-accessible amino acids of intact, folded proteins [5].

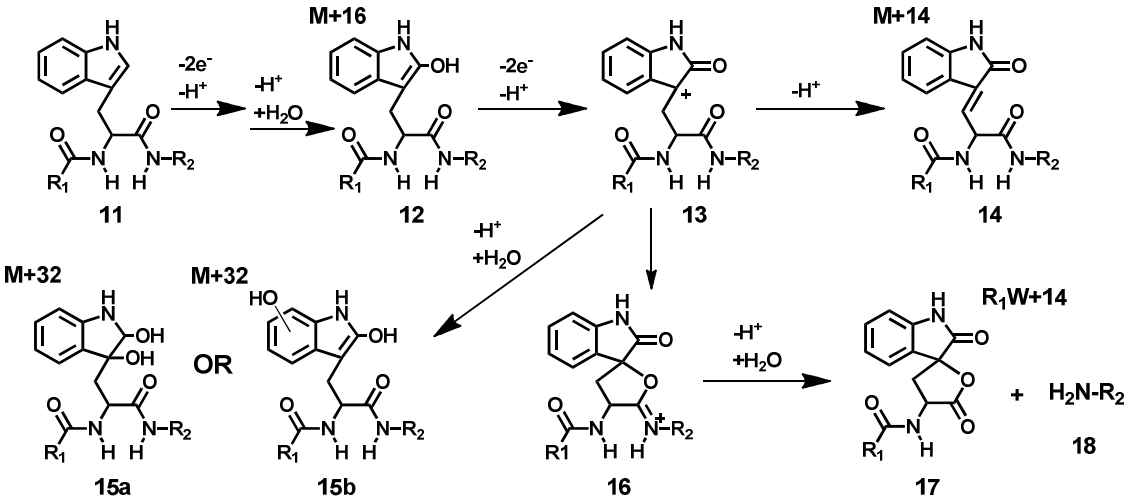
Electrochemical oxidation of peptides and proteins combined with mass spectrometry (MS) has revealed specific cleavage of the peptide bond at the C-terminal side of Tyr and Trp residues [6,7]. In proteomics experiments, the protein digestion method of choice is usually enzymatic, and particularly tryptic, but in specific cases chemical cleavage is used, for example with cyanogen bromide after Met [8,9], when enzymes do not have the required specificity. Electrochemistry may offer an interesting instrumental alternative to chemical and enzymatic cleavage, with several advantages such as speed of analysis, easy coupling of the electrochemical flow cell to MS or LC-MS and the distinct amino acid specificity.

The development of an automated proteomics method, based on electrochemistry, requires further improvement in reproducibility and cleavage yield. Although cleavage has been observed in most analyzed peptides and proteins [6,7], yields varied considerably and non-cleavage oxidation products were, in most of the cases, more abundant. We have studied several experimental parameters (adjacent amino acid, supporting electrolyte, pH) to get a better insight into the limiting steps on the way to an improved cleavage yield. The

study of the reaction mechanism and the characterization of reaction products by on-line EC-MS and LC-MS/MS extend the initial work on cleavage reactions [7,10-14] and facilitate the further development of electrochemical oxidation and cleavage of peptides and proteins as an analytical tool.



Scheme 1 Proposed electrochemical oxidation and cleavage pathways of Tyr-containing peptides (adapted from Permentier et al. [6]).



Scheme 2 Proposed electrochemical oxidation and cleavage pathways of Trp-containing peptides (adapted from Permentier et al. [7]).

2. Materials and Methods

2.1 Chemicals

Tyr and Trp-containing tripeptides (LYL, GYG, EYE, KYK, LWL, GWG, EWE, KWK) were obtained from Research Plus Inc. (Barnegat, NJ, USA). Formic acid (HCOOH), trifluoroacetic acid (TFA) and lithium hydroxide (LiOH) were obtained from Sigma Aldrich (Steinheim, Germany). Water was purified by an Arium Ultrapure water system (conductivity 18.2 MΩ.cm, Sartorius Stedim Biotech, Göttingen, Germany). HPLC supra gradient acetonitrile, glacial acetic acid (CH_3COOH) and lithium chloride (LiCl) were purchased from Merck (Darmstadt, Germany).

Stock solutions of Tyr- and Trp-containing tripeptides were prepared in 90/10/1 (v/v/v) ultra-pure water/acetonitrile/formic acid at a concentration of 1 mM, and used to prepare fresh samples shortly before performing the experiments described below.

2.2 On-line Electrochemistry-Mass Spectrometry (EC-MS)

Stock solutions of LYL, GYG, EYE, LWL, GWG, EWE and KWK were diluted to a final concentration of 2 µM for the on-line EC-MS experiments. KYK was diluted to a final concentration of 10 µM due to the relatively low MS signal intensity.

The tripeptide solutions were oxidized at a flow-rate of 50 µL/min (syringe pump, KD Scientific Inc., Holliston, MA, USA) with a Coulochem 5021 conditioning cell (ESA Inc., Bedford, MA, USA) with a porous graphite flow-through working electrode, a palladium auxiliary electrode and a palladium reference electrode. The auxiliary and reference electrodes are located both up-stream and down-stream with respect to the working electrode.

On-line EC-MS experiments were done by linearly ramping the cell potential from 0 to 1400 mV at 2 mV/s (triplicate experiments) with a home-made potentiostat controlled by a MacLab system (ADInstruments, Castle Hill, NSW, Australia) and EChem software

(eDAQ, Denistone East, NSW, Australia). The oxidation products were directly monitored by mass spectrometry with an API365 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Ontario, Canada) upgraded to EP10+ (Ionics, Bolton, Ontario, Canada) and equipped with a TurboIonSpray source. The delay time of 35 s between product formation within the electrochemical cell and product detection within the mass spectrometer was taken into account when correlating detected products and cell potential. The oxidation yields were calculated based on the decrease of the extracted ion current of the unoxidized tripeptides.

2.3 Off-line EC-LC-MS experiments

2.3.1 Stability study of LYL and LWL oxidation products

Oxidation of the tripeptides prior to liquid chromatography-mass spectrometry (LC-MS) was performed off-line. Stock solutions of LYL and LWL were diluted to 10 µM in 90/10/1 (v/v/v) water/acetonitrile/formic acid, oxidized at constant potential, collected, and diluted two times with water to decrease the acetonitrile concentration to 5% for LC-MS analysis. LYL was oxidized at 1000 mV and LWL at 700 mV vs. Pd/H_2 and the currents recorded at the working electrode were around 20 µA and 5 µA respectively.

The reaction mixtures obtained after oxidation of LYL and LWL were analyzed by LC-MS at three different time points i.e. t = 0, t = 24 h and t = 1 week to assess the stability of the oxidation products. The samples were stored at room temperature for the duration of the study.

2.3.2 Experiments with different electrolytes at different pH values

Stock solutions of LYL and LWL were prepared in 90/10 (v/v) acetonitrile/water at a concentration of 10 µM. The supporting electrolytes (formic acid, trifluoroacetic acid, acetic acid, lithium chloride and lithium hydroxide) were added to a final concentration of 250 mM. The pH of the samples was measured with a Metrohm Herisau E512 pH-meter. The tripeptides LYL and LWL were oxidized at 1000 mV and 700 mV, respectively, and

the experiments repeated three times. At these potentials both LYL and LWL showed significant electrochemical conversions at each pH tested. The obtained reaction mixtures were diluted twofold with water and stored at room temperature before LC-MS analysis. The currents obtained at the working electrode for LYL and LWL, respectively, were: 20 µA and 5 µA for formic acid, 180 µA and 10 µA for trifluoroacetic acid, 30 µA and 6 µA for acetic acid, 500 µA and 600 µA for lithium chloride, 1000 µA and 60 µA for lithium hydroxide.

2.4 Liquid Chromatography - Mass Spectrometry (LC-MS)

Liquid chromatography was performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) equipped with an Ultimate gradient pump and Famos autosampler. A Vydac RP-C_{18} column (150 mm × 1 mm i.d., 5 µm particles, 300 Å pore size, Grace Vydac) was used for chromatographic separation at a flow rate of 50 µL/min. Mobile phase A consisted of ultra-pure water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid.

For analysis of the tripeptide-derived reaction products, 100 µL injections were performed and separation was achieved with a gradient of B (5-50% at 1 %/min). The column was directly coupled to an API 365 EP10+ triple quadrupole mass spectrometer for product detection in positive ion mode.

2.5 High resolution MS/MS

High resolution MS/MS experiments were performed on an LTQ-Orbitrap XL with HCD cell (Thermo Scientific, Bremen, Germany). On-line nano-HPLC separation was performed with an easy-nLC system (Proxeon, Odense, Denmark) equipped with a 75 µm i.d. x 15 cm Acclaim PepMap100 C_{18} column (Dionex, Amsterdam, The Netherlands). A 30 min gradient of 5-50 % acetonitrile in water/0.1 % formic acid was used at a flow rate of 300 nL/min.

Unoxidized and oxidized samples of 1 μM LYL and LWL (oxidized at 1000 mV and 700 mV, respectively) were prepared as above, and 5 μL were injected for LC-MS/MS analysis. MS spectra were recorded at a resolution of 60,000 followed by data-dependent acquisition of MS/MS spectra at a resolution of 30,000 after collision-induced fragmentation in the HCD cell.

3. Results and Discussions

3.1 Influence of adjacent amino acids on oxidation and cleavage of Tyr and Trp-containing tripeptides

The range of electrochemically oxidized peptides and proteins reported by Permentier et al. [6,7] encompasses Tyr and Trp residues in a wide variety of sequence environments, which makes it difficult to assess the influence of adjacent residues. Size and charge effects of adjacent residues could have a significant effect on both oxidation (access to the electrode surface) and subsequent cleavage (intramolecular freedom of movement). To determine their influence on the oxidation reaction and yield of reaction products, a set of tripeptides with Tyr or Trp flanked at both sides by leucine, glycine, glutamic acid or lysine was studied to investigate the effect of hydrophobic, acidic or basic amino acid residues N- and C-terminal to the electroactive Tyr or Trp. The small tripeptides have the advantage that they do not suffer from adsorption issues with the porous graphite electrode that were encountered with larger peptides [6] and thus reliably reflect the products formed within the cell.

Figure 1 shows the oxidation and cleavage yields of both sets of tripeptides, at 1000 mV for Tyr-containing peptides and at 700 mV for Trp-containing peptides, respectively. Potential-ramping experiments showed that optimal cleavage product intensity was observed at these potentials (see Figure 2). Results are reported as normalized signal intensities extracted from the ramping experiments at the two potentials and provide a reasonably good estimation of relative product abundances since the ionization efficiencies of the products are not expected to differ substantially compared to the unoxidized tripeptides (Figure 2). In the on-line EC-MS experiments the delay between product formation inside the electrochemical cell and product detection inside the mass spectrometer was 35 s. This has the advantage that all products formed after electrochemical oxidation were analyzed shortly after their formation, thus reducing the possibility of degradation or further chemical reaction.

The detected products can be divided in three groups: cleavage products, non-cleavage oxidation products for which a structure has been proposed (Schemes 1 and 2) and so far unassigned products, which include dimers for Tyr-containing peptides.

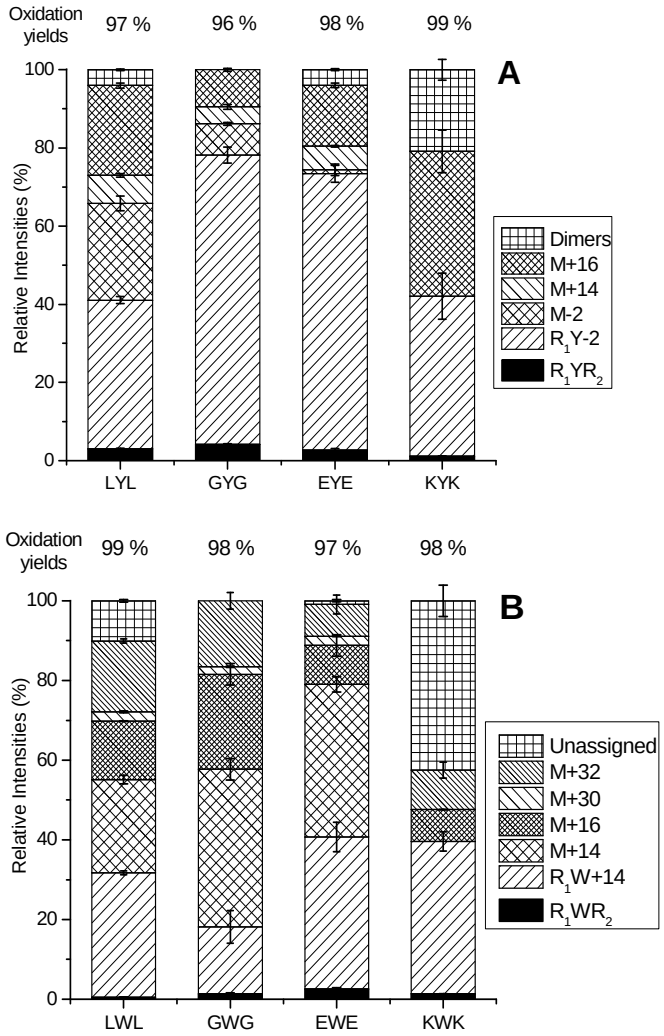


Figure 1 Relative ion intensities of oxidation and cleavage products obtained after on-line EC-MS experiments of A) LYL, GYG, EYE at 2 µM and KYK at 10 µM and B) LWL, GWG, EWE and KWK at 2 µM. All samples were oxidized in $H_2O/CH_3CN/HCOOH$ (90/10/1) by ramping the potential from 0 to 1400 mV at a scan rate of 2 mV/s. Ion intensities obtained at 1000 mV for Tyr-containing peptides and 700 mV for Trp-containing peptides were extracted and normalized to the total intensity of all the product peaks present in the spectrum (variation between 3 repetitions is shown with error bars). Oxidation yields obtained for each tripeptide are given on top of each graph.

All tripeptides were oxidized for more than 95% in each experiment based on the decrease of the extracted ion current of the unoxidized tripeptides (Figure 1). This indicates a very high conversion, presumably due to the high surface area of the porous graphite flow-through electrode and the small size of the peptides, which brings the electroactive groups into intimate contact with the electrode. However, the cleavage yields of Tyr-containing peptides, estimated by the relative intensities of the R_1Y-2 product (Scheme 1, structure **9**), together with the distribution of the non-cleavage oxidation products varied for the different peptides. At 1000 mV, LYL and KYK showed cleavage yields around 40% whereas higher yields (approximately 80%) were obtained for both GYG and EYE (Figure 1A). A lower steric hindrance with glycine (no side chain) compared to the bulky leucine and lysine may explain the higher cleavage yields for GYG, since cleavage of the peptide bond requires an intramolecular reaction that involves the carbonyl moiety of the following amide bond (Scheme 1) but results with EYE indicate that this is not the only factor. The high cleavage yield of EYE may be related to the observation that an acidic environment facilitates cleavage. The ratio between non-cleavage oxidation products varied as well for the different peptides. Formation of the M-2 dienone (**3**), obtained by secondary oxidation of the phenoxonium intermediate (**2**), was favored only for the LYL tripeptide whereas the hydroxylated forms M+14 and M+16 were the major by-products for GYG, EYE and KYK. Pyroglutamic acid, which is formed by cyclization of glutamic acid accompanied by loss of water (-18 Da), was observed for both cleaved EY-2, thus detected as EY-16, and hydroxylated EYE, and was independent of the applied potential.

Lower cleavage yields were reached with the set of Trp-containing peptides (Figure 1B). At 2 µM and 700 mV, yields ranging from 30% to 40% were estimated based on the amount of the R_1W+14 product and its hydroxylated equivalent R_1W+30 (see below) for LWL, EWE and KWK whereas a yield of around 20% was obtained for GWG. The lower cleavage yields may be related to the fact that cleavage is preceded by a hydroxylation step in case of Trp (Scheme 2). The resulting bulky oxo-indole group may explain why in contrast to Tyr no increased cleavage of glycine or glutamic acid was observed for Trp-containing tripeptides. In addition, competing non-cleavage reactions were favorable. In the case of Trp the non-cleavage oxidation products were mainly observed as the singly

hydroxylated M+14 and M+16 products, but low amounts of multiply hydroxylated Trp were also detected, already at the relatively low potential of 700 mV. Although the same types of cleavage and non-cleavage oxidation products were observed for all tripeptides, the ratios of oxidation and cleavage products are peptide-dependent. Tyr-containing peptides gave rise to higher cleavage yields, and the adjacent amino acid influences the secondary chemical reactions occurring after the initial oxidation step. The observed amino-acid dependence of cleavage yields indicates that oxidative protein cleavage *in vitro* or *in vivo* is not random.

3.2 Mechanism of electrochemical oxidation of LYL and LWL

3.2.1 On-line EC-MS: potential dependence of product formation

LYL and LWL were chosen for further study by on-line EC-MS, LC-MS and LC-MS/MS, since their products showed adequate retention during reverse phase chromatography (see Figure 3A-F) and because they generate the whole range of oxidation and cleavage products. Figure 2 shows the extracted ion voltammograms of LYL and LWL where product formation was followed as a function of cell potential. The sum of the ion intensities of the oxidation products is approximately equal to that of the unoxidized tripeptides. Oxidation of LYL starts at a potential of 500 mV as indicated by a decrease of signal intensity of unoxidized LYL and a concomitant increase in the signals of the various oxidation products. The N-terminal Tyr cleavage product, for which a spirodienolactone structure has been proposed (Scheme 1, structure **9**), was observed as LY minus 2 Da (LY-2). The C-terminal cleavage product, unmodified Leu (**10**), was also detected. The main non-cleavage oxidation products in Figure 2A correspond to hydroxylated Tyr (mass increase of 16 Da (M+16), **4a, 4b, 5**) and to hydroxylated Tyr that is further oxidized to the benzoquinone (**6**) (M+14) as well as a product with a mass decrease of 2 Da (M-2) corresponding to the dienone (**3**). Product distribution changes as the potential increases. Notably dimer formation, probably via Tyr crosslinking [15], was observed at potentials above 1000 mV for LYL.

The onset of oxidation of Trp in LWL also occurs at 500 mV, as indicated by the decrease of signal intensity for LWL (Figure 2B), confirming that Trp and Tyr have similar oxidation potentials in strongly acidic solutions [16]. In our previous studies [7], Trp cleavage products from proteins contained an additional oxygen, assumed to be due to hydroxylation of the indole moiety prior to spirolactone formation (Scheme 2, (**17**)). This suggests that Trp must be hydroxylated (**12**) before the cleavage reaction can occur. Hydroxylation was indeed observed for all Trp-containing tripeptides, and the major cleavage product was the LW+14 Da form. However, detailed analysis of oxidation products by on-line EC-MS revealed small amounts of other LWL cleavage products. Two products with *m/z* values of 318 and 348 (LW+30) were detected between 500 mV and 800 mV over a narrow potential window. The *m/z* 318 product indicates that cleavage of the tryptophanyl bond can also occur in the absence of a prior hydroxylation step, albeit to a minor extent. The equivalent product was however not detected for the other three Trp-containing peptides. The presence of the *m/z* 348 (LW+30) ion indicates the formation of a doubly hydroxylated Trp-containing N-terminal cleavage product. This cleavage product was found in low amounts in all Trp-containing tripeptides indicating that the Trp indole group was still reactive after electrochemical cleavage. However, neither the non-hydroxylated, nor the multiply hydroxylated cleavage products were detected in electrochemical protein digests [7], suggesting that their yield is usually very low.

On-line EC-MS also allowed detailed analysis of the non-cleavage oxidation products after oxidation of Trp-containing peptides, an area that has not been covered in previous studies [6,7]. The major products comprised singly- and multiply-hydroxylated Trp residues with up to 4 hydroxylation sites. This is in accordance with previous work on Trp oxidation through chemical methods [17-19]. Figure 2B shows the signal intensities of the products with up to 4-times hydroxylated Trp (M+16, M+32, M+48 and M+64). Although singly (M+16) and doubly (M+32) hydroxylated products were formed at the onset of Trp oxidation, their intensity decreased at potentials above 800 mV when further hydroxylated products (M+48 and M+64) were formed. A similar decrease of signal intensity was observed for the cleavage product (LW+14) at potentials greater than 900 mV. However, the products that were detected at potentials above 1000 mV did not compensate for the

decrease of LWL. It is thus possible that further products were formed that were not detected by mass spectrometry due to poor ionization.

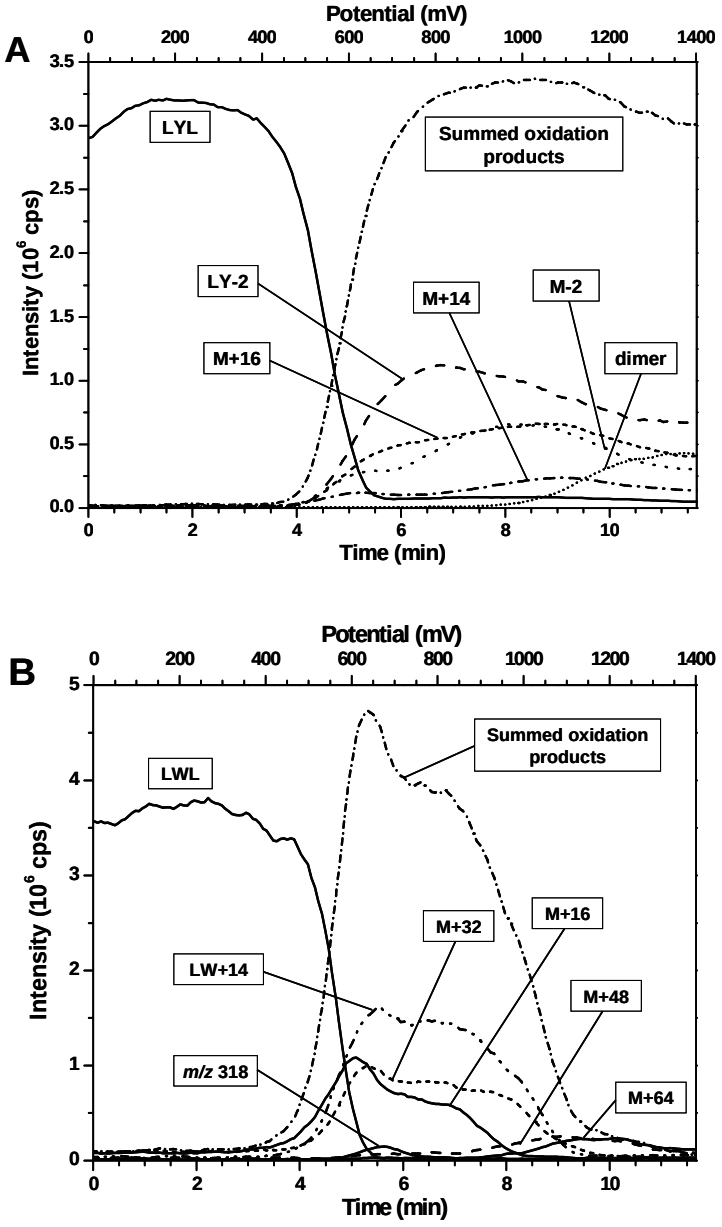


Figure 2 On-line EC-MS voltammograms of A) 2 µM LYL and B) 2 µM LWL in $H_2O/CH_3CN/HCOOH$ (90/10/1). The potential was ramped from 0 to 1400 mV with a 2 mV/s scan rate. Traces of the most abundant products were extracted and plotted versus time and cell potential. The sum of intensities of all the oxidation and cleavage products is indicated in the voltammogram.

On-line EC-MS provides a detailed insight into the reactions of Tyr- and Trp-containing peptides at various potentials and allows almost real-time monitoring of even potentially unstable products. However, isobaric compounds cannot be distinguished, and it cannot be excluded that matrix effects (e.g. ion suppression) hamper the accurate determination of product yield, that can be better evaluated by off-line EC followed by LC-MS analysis.

3.2.2 LC-MS of electrochemically oxidized tripeptides

Separation of oxidation products by reverse phase chromatography can overcome ion suppression effects that may be encountered during on-line EC-MS thus providing additional information about the range of oxidation products and, consequently, about the reaction mechanism. Unstable products may, however, escape analysis, so both approaches should be employed for a comprehensive analysis.

Comparison of LC-MS chromatograms of unoxidized and oxidized tripeptides showed that all reaction products observed by on-line EC-MS were detected. Several isobaric products were detected as multiple LC peaks, but no additional, major products with distinct masses appeared that might have gone undetected by EC-MS. Figure 3A shows the extracted ion chromatograms (XICs) of reaction products of LYL after oxidation at a constant potential of 1000 mV. Although the mixture of oxidation products was directly injected after off-line oxidation, the relative intensity of product peaks was, to some extent, different from that obtained by on-line EC-MS (Figure 2A). This may be due to the different ionization conditions but more likely to the limited stability of some of the reaction products, since the delay between oxidation and analysis was at least 30 min in off-line EC-LC-MS.

The M+16 non-cleavage oxidation product eluted in three separate peaks (M+16 (a), (b) and (c)) suggesting that three different isomers of this compound are generated corresponding to the ortho (**5**),meta (**4a**) and para (**4b**) hydroxylated Tyr derivatives represented in Scheme 1. The M-2 oxidation products eluted in two peaks (M-2 (a) & M-2

(b)) indicating the presence of two isomers. The major M-2 (a) peak has been identified by tandem MS as being the dienone product **3** (Figure 4A & MS/MS section) whereas the very weak second peak might be the intermediate (**8**) (Scheme 1) that is formed after nucleophilic attack of the carbonyl oxygen on the phenoxonium cation (**2**). The M+14 product (**6**) coeluted with one of the M+16 products and its abundance was notably decreased as compared to the on-line EC-MS experiment (Figure 2A), where the intensity of the M+14 product peak was about half that of the M+16 product.

Stability of oxidation products, in particular of the cleavage products, is very important for development of a reliable protein cleavage application. Reaction products were reanalyzed after 24 h (Figure 3B) and after 1 week at room temperature (Figure 3C). Both the M-2 and the M+16 (c) products disappeared after 24 h (Figure 3B and 3C). The minor M-2 (b) product, assigned to the cleavage intermediate (**8**), might further react towards cleavage. However, disappearance of the major M-2 (a) and the M+16 (c) product did not lead to equally intense new peaks. A concomitant increase in intensity of the first eluting M+16 peak (M+16 (a)) and the detection of a new peak with a mass increase of 30 Da (M+30) suggests reactions in solution, presumably with water. M-2 (a) may well react with water to form hydroxylated Tyr, but it is not clear how the M+30 product, presumably a hydroxylated form of M+14, may be produced from the M-2 or M+16 products. These observations stress the importance for on-line EC-MS in addition to LC-MS to obtain a complete picture of the reaction products. We can, however, not exclude that highly unstable products, which react further within seconds, escaped also on-line EC-MS. The LY-2 cleavage product was stable in solution, although hydrolysis of the spirolactone occured after extended storage at pH 2.4 at room temperature, as indicated by the formation of the *m/z* 311 product after 1 week (Figure 3C), which corresponds to hydrolyzed LY-2 with a mass increase of 18 Da.

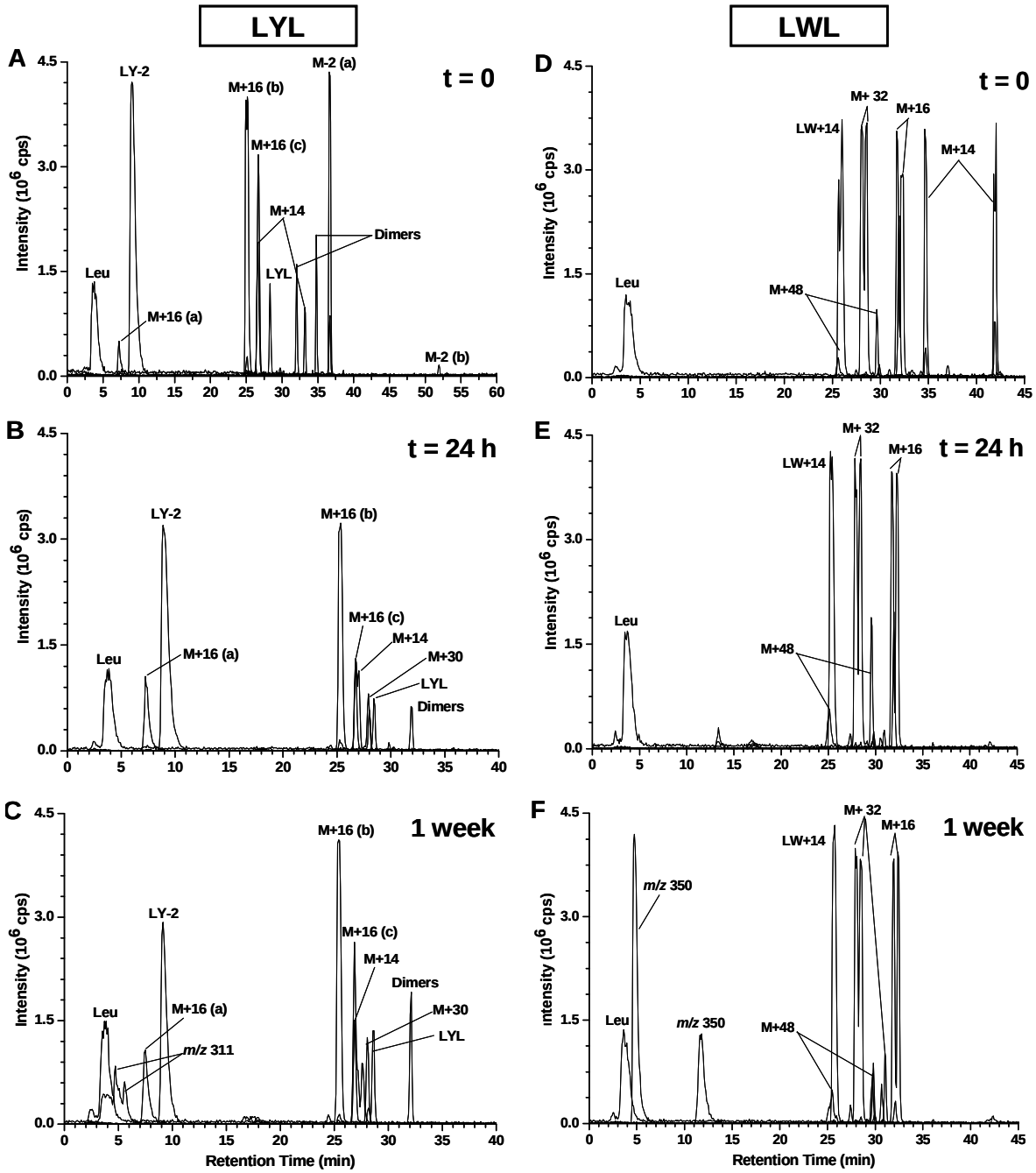


Figure 3 LC-MS analyses of oxidation products of LYL (A, B, C) and LWL (D, E, F); A and D: directly after electrochemical oxidation; B and E: after storage for 24 hours; C and F: after storage for 1 week at room temperature. Electrochemical oxidation was performed at a peptide concentration of 10 µM in $H_2O/CH_3CN/HCOOH$ (90/10/1) at a constant potential of 1000 mV for LYL and 700 mV for LWL and a flow-rate of 50 µL/min.

Figures 3D, E and F show the XICs of the reaction products of LWL directly after oxidation at a constant potential of 700 mV, and after storage. Multiple hydroxylation of the indole group, which is detected at very low yields in on-line EC-MS, is clearly evidenced in LC-MS by the detection of different M+32 products (Figure 3D). Similarly to LYL, several isomers of the different hydroxylated products were observed (two M+14, two M+16 and two M+32 LC peaks) pointing to several hydroxylation sites. Only the M+14 products appeared to be unstable, since they were not detected after 24 h storage at room temperature (Figure 3E). Hydrolysis of the spirolactone of the cleavage product LW+14 was also observed after storage for a week at room temperature at pH 2.4 (Figure 3F) as reflected by the formation of a product with an 18 Da mass increase to *m/z* 350.

LC-MS analyses of reaction mixtures generated after electrochemical oxidation of Tyr- and Trp-containing peptides indicate that they are more complex than expected based on on-line EC-MS due to a number of isobaric compounds. Some oxidation products disappeared already after 24 h, but the cleavage products were stable for at least a day. Extended storage led to spirolactone hydrolysis but not to further degradation of the cleavage products.

3.3.3 MS/MS characterization of reaction products

Both low and high resolution MS/MS measurements were performed to further characterize some of the oxidation and cleavage products. Figure 4A shows the MS/MS spectrum of the *m/z* 406 peak for which an M-2 structure has been proposed (Scheme 1, structure **3**). A fragment at *m/z* 300 indicates a neutral loss of 106 Da that corresponds to loss of the dienone moiety as p-quinomethane. The presence of the corresponding y_2 and b_2 ions at *m/z* 293 and 275 respectively are also consistent with the proposed M-2 structure in Scheme 1. Figures 4B and 4C show the MS/MS spectra of the cleavage products assigned to LY-2 (**9**) and LW+14 (**17**) respectively. The fragmentation pattern of LY-2 (Figure 4B) confirms the proposed structure as testified by the loss of CH_2O_2 (*m/z* 247) from the spirolactone moiety and the further fragmentation of the amide bond yielding the Tyr-

derived immonium ion with a mass decrease of 2 Da due to the dienone structure of the modified Tyr (*m/z* 134). LW+14 (Figure 4C) fragments into the y_1 ion (*m/z* 219) which further loses CH_2O_2 (similarly to LY-2) from the proposed spirolactone moiety to yield the fragment observed at *m/z* 175. The latter corresponds to the Trp-derived immonium ion that has a 16 Da mass increase due to hydroxylation occurring prior to the cleavage reaction (Scheme 2). Loss of ammonia (NH_3) accounts for the two other major peaks observed in the MS/MS spectrum (Figure 4C). Figure 4D and 4E compare the MS/MS spectra of two isoforms of the M+16 oxidation products (M+16 (b) and M+16 (c) in Figure 3) generated from LYL. Clearly different fragmentation patterns were obtained for the two products that relate to the localization of the hydroxylation sites. The fragmentation pattern of M+16 (b) (Figure 4D) indicates that Tyr is hydroxylated at the para-position of the aromatic ring, since the b_2 ion (*m/z* 293) readily loses the aromatic OH group as water and CO to yield the *m/z* 247 fragment. Moreover the intense peak at *m/z* 134 corresponds to the immonium ion of modified Tyr with a dienone structure (Tyr - 2 Da). The second M+16 isoform (Figure 4E) was identified as LYL that is hydroxylated at the ortho-position of the aromatic ring. The presence of the fragment at *m/z* 152, corresponding to the Tyr immonium ion with a mass increase of 16 Da, confirms the presence of the hydroxyl group at the ortho-position of the aromatic ring, which is not as easily lost during fragmentation as the hydroxyl group in the para-position. The presence of the y_2 ion at *m/z* 311 and the b_2 ion at *m/z* 293 also fits the expected fragmentation pattern of an ortho-hydroxylated Tyr. Moreover, the b2 ion readily loses CO (m/z 265) followed by water to form an epoxide (m/z 247), as observed for other catechols (e.g. dopamine) [20].

High resolution MS/MS spectra allowed us to confirm the proposed structures for both Tyr and Trp cleavage products, a characterization that has, to our knowledge, not been performed in earlier studies. The assignment of the hydroxylation sites in the two Tyr M+16 isoforms is in agreement with the reaction products shown in Scheme 1.

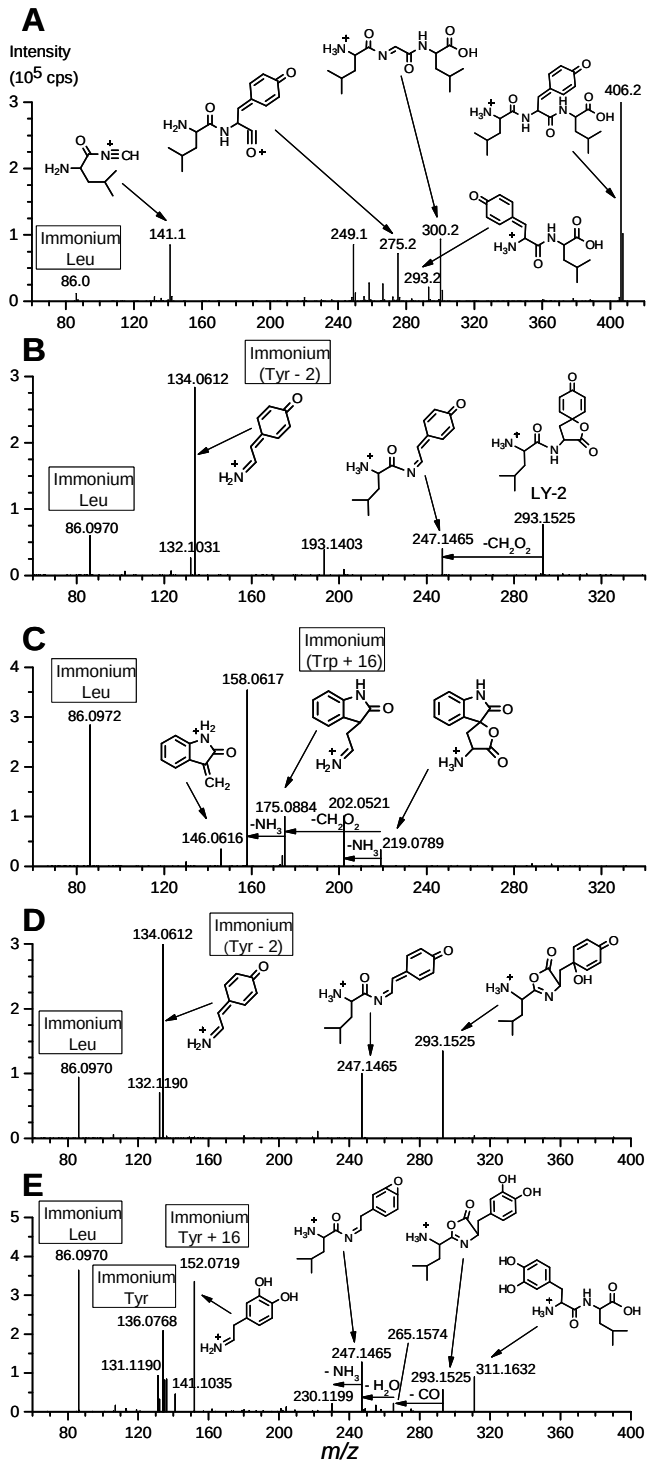


Figure 4 MS/MS analysis of oxidation and cleavage products of LYL and LWL. A: triple quadrupole product ion spectrum of LYL-2 (*m/z* 406); high resolution product ion spectra of (B) LY-2 (*m/z* 293), of (C) LW+14 (*m/z* 332) and of (D, E) two isobaric LYL M+16 oxidation products i.e. M+16 (b) (*m/z* 424) and M+16 (c) (*m/z* 424) (see Figure 3A).

3.3 Effect of the supporting electrolyte and the pH on oxidation and cleavage

The pH of the solution affects the protonation state of Tyr, which in turn affects its redox potential and the initial electrochemical oxidation steps. It is known that the oxidation potentials of Tyr and Trp decrease (approximately 60 mV/pH unit) when increasing the pH [16]. The pH of the solution also affects the potential applied at the working electrode due to pH sensitivity of the Pd/H_2 reference electrode: increasing the pH leads to a decrease of the actual potential of the working electrode by approximately 60 mV/pH unit at 293 K, as well, so that both effects compensate each other in the case of our study. In addition, the pH and solution electrolytes may affect the subsequent chemical reactions, including cleavage. To study these effects, oxidation of LYL and LWL tripeptides was performed in different media and the reaction mixture was subsequently analyzed by LC-MS. Several electrolyte compositions covering three pH regions were tested: strongly acidic (pH 1.9-3.1), weakly acidic (pH 5.2) and strongly basic (pH 12.2).

3.3.1 Strongly acidic conditions

Trifluoroacetic acid (TFA), formic acid and acetic acid were used as supporting electrolytes at a concentration of 250 mM resulting in pH values of 1.9, 2.4 and 3.1, respectively. For LYL at 1000 mV (Figure 5A), cleavage products were observed in all cases, with yields between 20% and 30%. Total oxidation yields were above 95%, as testified by the low amount of remaining, unoxidized LYL. Non-cleavage oxidation products (M-2, M+14 and M+16) represented about 60% of the total ion intensity with the M+16 oxidation products being most abundant. Five to 15% of the total ion intensity was assigned to other products, which are mainly related to dimers due to Tyr-Tyr crosslinking.

Even higher conversions of nearly 100% were obtained for LWL at 700 mV under strongly acidic conditions (Figure 5B). Cleavage yields, determined based on the L+14 product, were on the order of 30% and thus comparable to LYL. Singly or multiply hydroxylated products (up to 4 oxygen insertions) accounted for 60% of the total ion

intensity. A few unidentified products (5-15%) were observed, the structures of which could not be assigned due to their low abundance.

We conclude that the nature of the electrolyte at strongly acidic pH does not affect the oxidation and cleavage yields of LYL and LWL significantly. We thus prefer the use of formic acid as supporting electrolyte, since it is most suitable for electrospray ionization of peptides.

3.3.2 Weakly acidic pH conditions

Oxidation of LYL and LWL was performed under weakly acidic conditions in the presence of 250 mM lithium chloride at pH 5.2 where the phenolic OH group of Tyr (pK_a 10.1) is still in its uncharged state. The effect of pH and of the electrolyte (no proton donor) on the secondary chemical oxidation can thus be compared to the one obtained under strongly acidic conditions, since the redox potential (and thus the oxidation yield) is not expected to change between the two conditions [16]. We observed, however, an increase of pH, from 5.2 to 8.8 for LYL and from 5.2 to 6.6 for LWL, measured before and after the electrochemical cell. The change of pH is explained by reduction of water at the counter electrode, combined with the low buffer capacity of the LiCl solution. Reactions at counter electrode could potentially influence the peptide (products) as well but we expect these reactions to have a minor effect due to the much larger active surface area of the porous graphite working electrode. Figure 5 shows that switching from strongly acidic to weakly acidic starting conditions only slightly affected the nature and distribution of the oxidation products, although a large variation in product intensities in repeated experiments was observed for the Tyr-containing peptides. The oxidation yield decreased to 85% for LYL (Figure 5A), but the cleavage yield did not decrease appreciably and was still around 20%. The large increase in pH observed for LYL may have contributed to the lower conversion of the peptide. In contrast, the oxidation yield of LWL was not affected under these conditions, and was close to 100% (Figure 5B). However, cleavage was significantly reduced to 15% compared to 30% under strongly acidic conditions. The simultaneous increase of doubly and triply hydroxylated products indicates that Trp-hydroxylation is favored under weakly acidic

conditions, and thus not favorable to increase electrochemical cleavage of Tyr- and Trp-containing tripeptides.

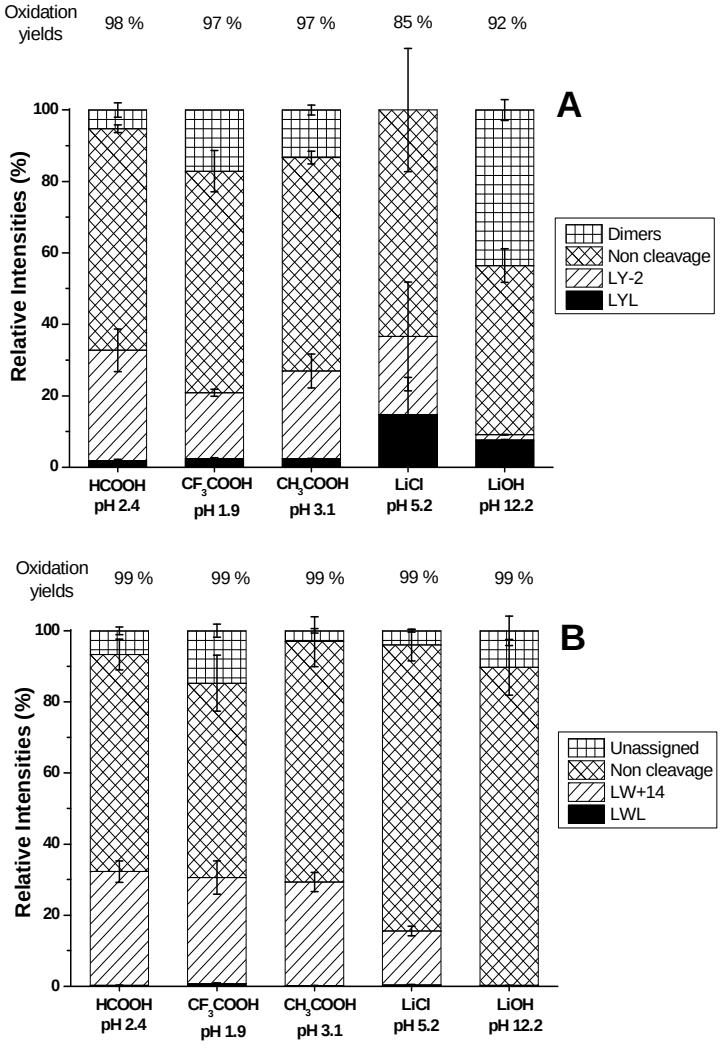


Figure 5 Comparison of the abundance of reaction products after electrochemical oxidation of A) LYL at 1000 mV and B) LWL at 700 mV in the presence of different electrolytes and at different pH values. Abundances were determined by integration of extracted ion chromatograms after LC-MS analysis and normalization to the total intensity of all the product peaks present in the spectrum (variation between 3 repetitions is shown with error bars). Oxidation yields obtained for each condition are given on top of each graph.

3.3.3 Strongly basic conditions

It is conceivable that the ratio between oxidation and cleavage products of LYL and LWL is affected by switching to strongly basic pH conditions, since the spirodienolactone moiety is more rapidly hydrolyzed at basic pH [13,21]. Lithium hydroxide was used as supporting electrolyte at a concentration of 250 mM giving a pH value of 12.2 for LYL and LWL, which is significantly higher than the pK_a of 10.1 of the phenolic OH group in Tyr and of subsequent aromatic hydroxylation products of Tyr or Trp.

Working under strongly basic conditions significantly affects oxidation of Tyr and Trp as well as the subsequent chemical reactions of the reactive intermediate cations. Reduced oxidation yields of around 90% were obtained for LYL (Figure 5A) indicating reduced charge transfer. Secondary reaction pathways are more drastically affected, since very little peptide bond cleavage (1-2%) was detected. The hydrolyzed form of the cleavage product was not detected either, confirming that the cleavage of the peptide bond did not occur. The M-2 non-cleavage oxidation product and LYL dimers were most abundant. Combined with the absence of hydroxylated oxidation products and the very low amount of cleavage products this suggests that nucleophilic attack at the phenoxonium intermediate (see Scheme 1) is not favored under basic conditions. For LWL, initial electrochemical oxidation of Trp is not affected at strongly basic pH (see Figure 5B). Similarly to LYL, no cleavage products, neither in the LW+14 nor in the hydrolyzed form, were observed under these conditions. Hydroxylated, non-cleavage oxidation products (M+14, M+32 and M+48) dominated the mass spectrum.

Basic pH is thus not favorable either for oxidative peptide bond cleavage after Tyr or Trp. Although oxidation yields are still good, formation of the spirolactone intermediate, that precedes hydrolysis of the peptide bond, is strongly disfavored.

4. Conclusion

Electrochemical, oxidative cleavage of peptide bonds C-terminal to Tyr and Trp has been studied in detail by varying the adjacent amino acids, pH and electrolyte. Combination of on-line EC-MS and off-line EC-LC-MS(/MS) provided complementary information. On-line EC-MS covers some of the unstable reaction products while LC allows to separate isobaric oxidation products prior to MS(/MS). LC-MS(/MS) permits also a more accurate determination of reaction yields and product distribution. On-line EC-MS allowed studying product formation as a function of cell potential, which must be controlled in order to limit unwanted side products, such as Tyr-Tyr dimers.

Our results show that the highest cleavage yields were obtained when working under strongly acidic conditions (pH 1.9-3.1) and that no or little peptide backbone cleavage was detected at basic pH (pH 12.2). Cleavage yield reached up to 80% for GYG, a peptide with minimal steric hindrance. Electrochemistry provides lower overall cleavage yields than enzymatic and chemical methods due to competing non-cleavage oxidation reactions, which are difficult to avoid in view of the reaction mechanism. In order to overcome this limitation, we are currently investigating the possibility of separating the initial electrochemical oxidation and the subsequent chemical reactions. However, the clear advantage of electrochemical cleavage lies in the speed of analysis. Cleavage and oxidation products are formed within seconds in the electrochemical cell and can be directly detected by coupling to an (LC-)MS system. The technique is fully instrumental and thus has the potential for full automation, since oxidation and cleavage are induced and controlled entirely through electrical potentials.

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Chapter 4

Chemical Labeling of Electrochemically Cleaved Peptides

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Rapid Communications in Mass Spectrometry
27, (4), 546-552
2013

Abstract

Electrochemical oxidation of peptides and proteins can be performed in high yields with up to 100% conversion for small peptides. Electrochemical cleavage of peptide bonds specifically next to tyrosine (Tyr) and tryptophan (Trp) in the peptide/protein backbone reaches yields of 50% or more (up to 80% for certain small peptides under optimized conditions). Electrochemical oxidation reactions yield complex mixtures due to the generation of non-cleavage oxidation products in addition to cleaved peptides. In this work we describe a chemical tagging procedure that targets the reactive spirolactone groups that are generated at the newly formed C-terminus after electrochemical oxidation and cleavage next to Tyr and Trp. We use hexylamine which specifically reacts with the spirolactone moieties and show that working at pH 10 with a reaction time of 0.5 min leads to the highest conjugation yields in an aqueous environment. We subsequently show that the excess of hexylamine over spirolactone groups can be significantly decreased by working under non-aqueous conditions in pure acetonitrile to prevent spirolactone hydrolysis. The tagged cleavage products can be monitored by precursor ion scanning on a triple quadrupole mass spectrometer, which allows selective detection of the cleaved and tagged peptides.

1. Introduction

Proteomics is a challenging field of research that aims at identifying and quantifying proteins in complex biological matrices in order to relate proteomic changes to biological mechanisms. The widely accepted workflow in proteomics is based on protein digestion in a sample of interest and analysis of the resulting peptides by reversed phase liquid chromatography coupled to tandem mass spectrometry (RPLC-MS/MS). Proteins are finally identified by comparing experimental MS/MS spectra with *in silico*-generated spectra using database search algorithms.

Enzymatic digestion is currently the method of choice for cleaving proteins at specific peptide bonds. Although trypsin is the most commonly used enzyme, the proteomics toolbox contains several proteases with different amino acid specificities. Chemical cleavage is also used in specific cases, sometimes in combination with enzymatic digestion, notably when enzymes do not have the required specificity but the scope of site-specific chemical cleavage reagents with high yield is limited. Alternative cleavage methods are therefore of interest for specific applications.

Electrochemistry may offer an interesting instrumental alternative to chemical and enzymatic cleavage. Electrochemical oxidation of peptides and proteins combined with MS revealed specific cleavage of the peptide bond at the C-terminal side of tyrosine (Tyr) and tryptophan (Trp) residues [1,2]. However, electrochemical oxidation of peptides and proteins yields a complex mixture of cleavage and non-cleavage oxidation products that can be difficult to analyze.

A previous study explored experimental parameters to get a better insight into the limiting steps determining cleavage yield [3]. The highest cleavage yields were obtained under strongly acidic conditions and yields gradually decreased with increasing pH. Although up to 80% cleavage yield was observed for GYG, a small tripeptide with limited steric hindrance, electrochemical cleavage yields still lag behind enzymatic reactions when it comes to large peptides and proteins due to competing non-cleavage oxidation reactions.

Previous work showed that most of the studied proteins lead only to partial sequence coverage when using electrochemistry as a cleavage tool. In addition, a significant amount of oxidized, intact protein was observed [2].

An interesting aspect of electrochemically cleaved peptides is that the newly generated C-terminus is an acid stable spirolactone moiety [3] that may be targeted by chemical labeling. The reactivity of spirolactones has been studied for organic synthesis purposes and ring opening reactions have been shown to occur under specific conditions [4-6]. Chemically synthesized Tyr-spirolactones were, for instance, shown to readily undergo methanolysis at slightly basic pH, leading to a ring opening reaction at 0°C with formation of the corresponding hydroxy-Tyr methyl ester, and to a cyclization reaction producing a hydroindolenone at room temperature [4]. Aminolysis of esters and lactones is a general method for preparation of amides in synthetic organic chemistry. An example in peptide chemistry is the aminolysis of homoserine lactone, which is generated upon cleavage C-terminal to methionine residues by cyanogen bromide (CNBr), and reacts with primary amines at basic pH leading to the formation of a new amide bond [7-11]. Shi *et al.* recently developed a tagging method targeting homoserine lactone generated after CNBr cleavage of peptides to study protein-protein interactions by crosslinking. Polyhistidine and biotin tags were coupled via the formation of an amide bond resulting from the reaction of the primary amine with the lactone moiety [11].

We report here the use of a primary amine, hexylamine, to assess the specific tagging of spirolactone moieties at the C-termini of electrochemically cleaved peptides. The influence of pH, reaction time and tag concentration were investigated in order to optimize the tagging yield. Precursor ion scanning targeted at specific fragments was used to selectively detect hexylamine-tagged peptides within the mixture of the cleaved and non-cleaved electrochemical reaction products.

2. Material and Methods

2.1 Chemicals

Tyr- and Trp-containing tripeptides (LYL and LWL) were obtained from Research Plus Inc. (Barnegat, NJ, USA). LFL was purchased from Bachem (Weil am Rhein, Germany). Formic acid (HCOOH) and hexylamine 99% were obtained from Sigma Aldrich (Steinheim, Germany). Water was purified by an Arium Ultrapure water system (conductivity 18.2 MΩ.cm, Sartorius Stedim Biotech, Göttingen, Germany). HPLC supra gradient acetonitrile (CH_3CN), Dimethylformamide (DMF) and Dimethylsulfoxide (DMSO) were purchased from Biosolve (Valkenswaard, The Netherlands).

2.2 Electrochemical oxidation of peptides

Stock solutions of LYL and LWL were prepared in 89/10/1 (v/v/v) $H_2O/CH_3CN/HCOOH$ at a concentration of 1 mM and diluted to a final concentration of 10 µM prior to oxidation.

The tripeptide solutions were oxidized with a Coulochem 5021 conditioning cell (ESA Inc., Bedford, MA, USA) with a porous graphite flow-through working electrode and a palladium reference electrode at a flow-rate of 50 µL/min (syringe pump, KD Scientific Inc., Holliston, MA, USA). LYL was oxidized at 1000 mV and LWL at 700 mV vs. Pd/H_2 with a home-made potentiostat controlled by a MacLab system (ADInstruments, Castle Hill, NSW, Australia) and EChem software (eDAQ, Denistone East, NSW, Australia). The reaction products were collected for further labeling reactions.

2.3 Reaction with hexylamine

The mixture of electrochemically oxidized peptides (10 µM initial concentration) containing the cleavage products (200 µL, pH 2.8) reaches pH 4 upon addition of 800 µL of $H_2O/CH_3CN/HCOOH$ (89/10/1; v/v/v) and 20 µL hexylamine 99% (148.2 mM final concentration) in a glass vial. Experiments at high pH values were performed as follows: to 200 µL of the 10 µM peptide solution (220 µL for pH 10) was added a mixture of A:

hexylamine, B: $H_2O/CH_3CN/HCOOH$ (89/10/1; v/v/v) and C: 200mM sodium borate buffer pH 8.5. Volumes used for pH 8: 20 µL A, 500 µL B and 300 µL C; for pH 9: 20 µL A, 300 µL B and 500 µL C; for pH 10: 20 µL A, 300 µL B, 500 µL C plus 100 µL 1 M NaOH. All reactions were performed at room temperature with stirring. The reactions were quenched by the addition of 20 µL formic acid yielding acidic mixtures compatible with reversed phase LC-MS analysis.

Experiments under non-aqueous conditions were performed by drying 500 µL of the 10 µM peptide solution under nitrogen (20 min). 50 µL of a mixture of DMSO, DMF or acetonitrile and hexylamine (199:1) was added to the dried solution and allowed to react by vortexing for 30 sec at room temperature. The reaction was quenched by the addition of 1 µL formic acid and 450 µL ultrapure water prior to LC-MS analysis.

All experiments were performed in triplicate and peak areas obtained by LC-MS analyses were normalized to the peak area of 100 nM LFL that was added as internal standard to the mixture prior to LC-MS analysis. The normalized peak areas of the labeled products were normalized a second time with respect to the initial cleavage yields of LW+14 (for LWL) and LY-2 (for LYL), respectively, as these can vary by up to 50% between experiments.

2.4 Liquid Chromatography - Mass Spectrometry (LC-MS)

Liquid chromatography was performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) equipped with an Ultimate gradient pump and a Famos well plate Microautosampler. A Vydac RP-C_{18} column (150 mm × 2.1 mm i.d., 5 µm particles, 300 Å pore size, Grace Vydac) was used for chromatographic separation at a flow rate of 300 µL/min. Mobile phase A consisted of ultra-pure water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid.

For analysis of reaction products, 50 µL injections were performed, and separation was achieved with a gradient of 5-50% B at 1%/min. The column was directly coupled to an API365 triple quadrupole mass spectrometer (MDS-Sciex, Concord, Ontario, Canada)

upgraded to EP10+ (Ionics, Bolton, Ontario, Canada) and equipped with a TurboIonSpray source for product detection in positive ion mode.

2.5 MS/MS analysis and precursor ion scanning

Precursor ion scanning experiments were performed by monitoring the *m/z* 281.3 product ion for Tyr-labeled peptides and the *m/z* 320.2 product ion for Trp-labeled peptides. Collision energy and collision gas (CAD) settings were optimized for each compound. The following MS acquisition settings were used: *m/z* range 320-500, step size 1 amu, dwell time 10 ms.

3. Results

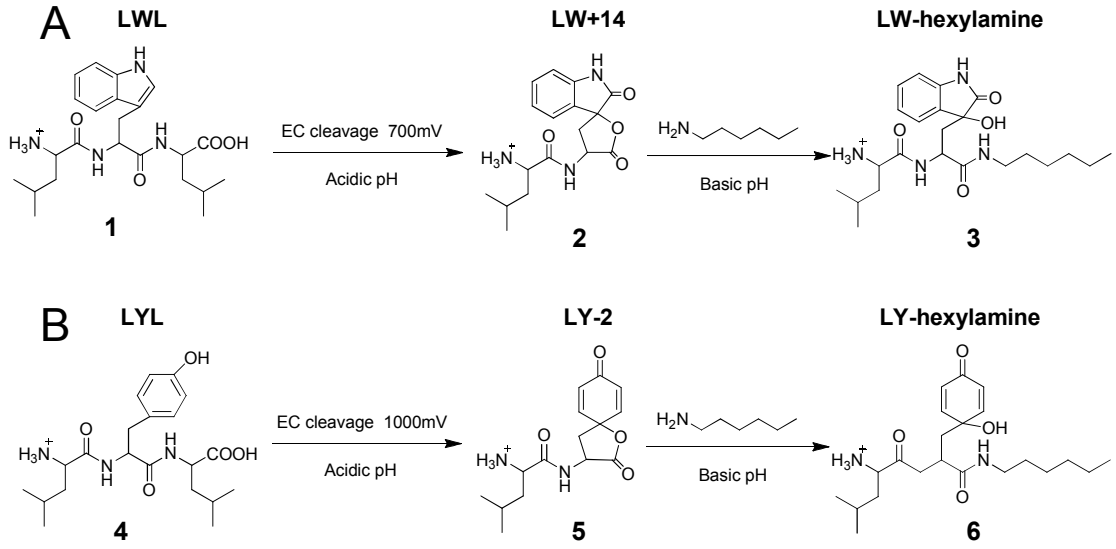
3.1 Hexylamine labeling of Tyr and Trp spirolactones

The tripeptides LWL (**1**) and LYL (**4**) (Scheme 1) were chosen to assess the feasibility of tagging the spirolactone moieties of electrochemically cleaved peptides. These tripeptides have been previously used to study the mechanistic aspects of electrochemical oxidation of Tyr- and Trp-containing peptides [3]. They generate oxidation and cleavage products in good yield and with well-characterized structures, and therefore are suitable test compounds to study the labeling reaction and potential side reactions. The tripeptides were oxidized with a porous graphite electrode at 700 mV (LWL) and 1000 mV (LYL), and the oxidized samples were collected for subsequent post-cell reactions.

Figure 1 compares the extracted ion chromatograms of the reaction products of LWL (**1**) and LYL (**4**) directly after electrochemical oxidation (panels A and C) with the chromatograms after reaction with a large excess of hexylamine (150 mM, 15,000 fold molar excess) at room temperature for 20 min at pH 8 (panels B and D). The cleavage products bearing spirolactone moieties (LW+14 (**2**), *m/z* 332, and LY-2 (**4**), *m/z* 293, for LWL and LYL, respectively) are both converted to a compound with a mass increment of 101 Da (*m/z* 433 (**3**) and *m/z* 394 (**6**), respectively), in agreement with aminolysis by hexylamine as shown in Scheme 1. No additional peaks corresponding to the addition of hexylamine to the non-cleavage products were detected (see Roeser et al. [3] for details about non-cleavage products). The overall decrease in intensity, especially in the case of LYL, is probably due to ion suppression by the excess of hexylamine which elutes throughout the chromatogram.

The labeled cleavage products, LW-hexylamine (**3**) and LY-hexylamine (**6**), were analyzed by MS/MS (Figure 2). The tagged compounds, which are dipeptides modified at their C-terminus, were expected to yield y- and b-type ions based on the predictable fragmentation pattern of peptides. Collision-induced dissociation of the parent compound LW-hexylamine (**3**) (*m/z* 433) yielded a y_1 fragment ion at *m/z* 320 which confirms the

reaction of hexylamine with the Trp-spirolactone whereas the b_2 ion at *m/z* 332 supports the formation of an amide bond as described in Scheme 1. The other fragments are also consistent with the expected structure of the labeled compound as shown in Figure 2A.



Scheme 1: Reaction of electrochemically generated spirolactones LW+14 (A) and LY-2 (B) with hexylamine under basic conditions.

The formation of LY-hexylamine (**6**) (*m/z* 394) is likewise confirmed by the presence of an intense y_1 ion at *m/z* 281 in the MS/MS spectrum (Figure 2B) and by the other assigned fragments. Both the LY- and the LW-hexylamine products elute at much longer retention times than their unreacted forms (Figure 1) due to the presence of the aliphatic hexyl group. Interestingly, both hexylamine-labeled cleavage products *m/z* 433 and *m/z* 394 elute as two distinct LC peaks (Figures 1B and 1D). The MS/MS spectra of both isoforms showed identical fragmentation patterns with only minor differences in peak intensities, and therefore did not give any further information on the nature of the difference between the two products: the MS/MS spectra in Figures 2A and 2B represent the early eluting peaks, while Figures 2C and 2D show the spectra of the later eluting isoforms. The

similarity in MS/MS spectra suggests that the isomers are structural conformers formed by the attack of hexylamine on either side of the spirolactone ring.

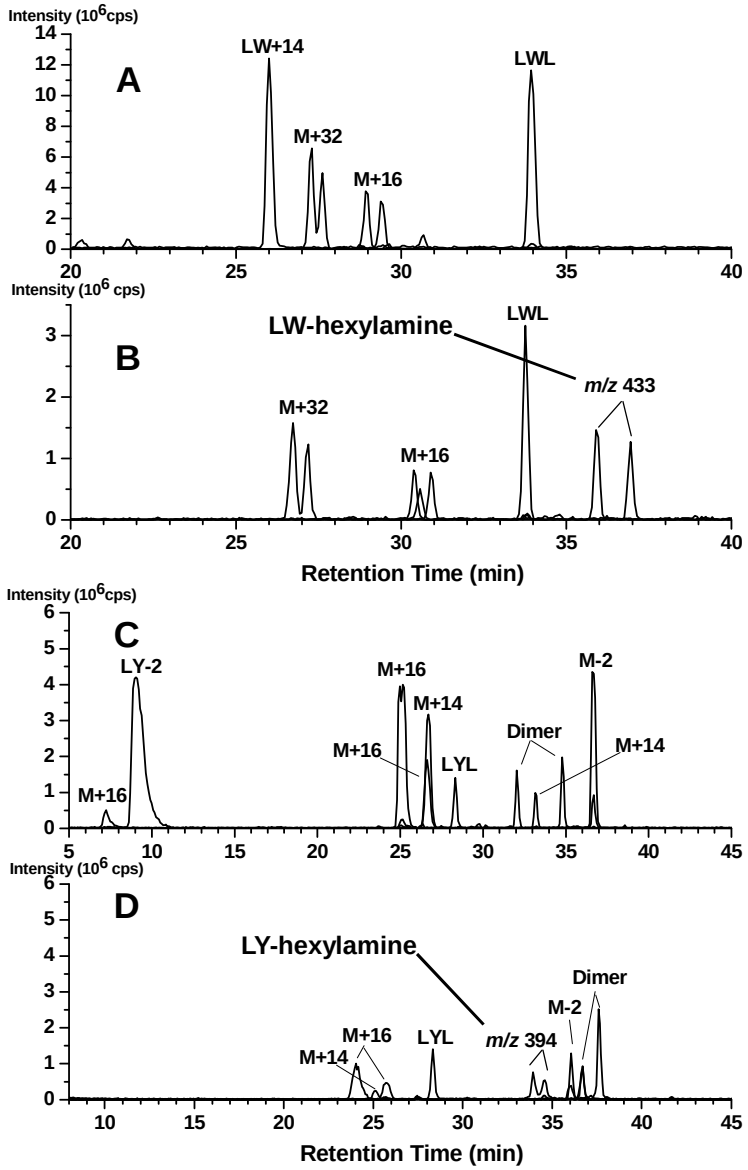


Figure 1: Combined extracted ion chromatograms of LWL and LYL reaction products after oxidation (panels A and B) and after reaction with a 15,000-fold molar excess of hexylamine for 20 min at room temperature in borate buffer pH 8 leading to the formation of the LW-hexylamine (panel C) and LY-hexylamine (panel D).

3.2 Influence of pH and reaction time

The effect of pH and reaction time on product yield was investigated in order to find the optimal conditions for the labeling of spirolactone residues under aqueous conditions. The moderate reactivity of the electrophilic spirolactone towards primary amines is advantageous as it reduces the possibility to react with primary amino groups that are present at the N-terminus of peptides or as part of the lysine side chain. The main competing reaction is hydrolysis of the spirolactone yielding the corresponding carboxylic acid [11].

Figures 3A and 3B compare the relative yields of labeled LW-hexylamine (**3**) and LY-hexylamine (**6**), respectively, at various pH values and reaction times. All experiments were performed with a 15,000-fold molar excess of hexylamine over spirolactone, to maximize the labeling yield. The pH region between 8 and 10 was studied and the amount of labeled products was compared to that obtained at pH 4 which corresponds to the pH value measured when only hexylamine was added to the reaction mixture (i.e. no buffer). Accurate reaction yields could not be determined since the unlabeled or hydrolyzed spirolactone products were not detected in the LC-MS analysis. Our conclusions are therefore based on the absolute intensities of the respective labeled compounds.

The reaction yields of hexylamine with the cleavage product LW+14 (**2**) increased with increasing pH and reached the highest yield at pH 10 (Figure 3A). Reaction time had only a minor influence and the maximal yield was almost reached after 0.5 min. Reaction times of 20 – 60 min resulted in the highest yield, while there was a slight but non-significant decrease after 120 min. The reaction kinetics of the LY-2 spirolactone (**4**) were quite different because the reaction product was not stable at pH 9 or 10 resulting in very low yields for reaction times of 20 min and longer (Figure 3B). The highest yield was obtained after 0.5 min reaction time at pH 10. Degradation of the Tyr-spirolactone or the labeled product by hydrolysis is likely to occur under these conditions but no new products were observed during the analyses.

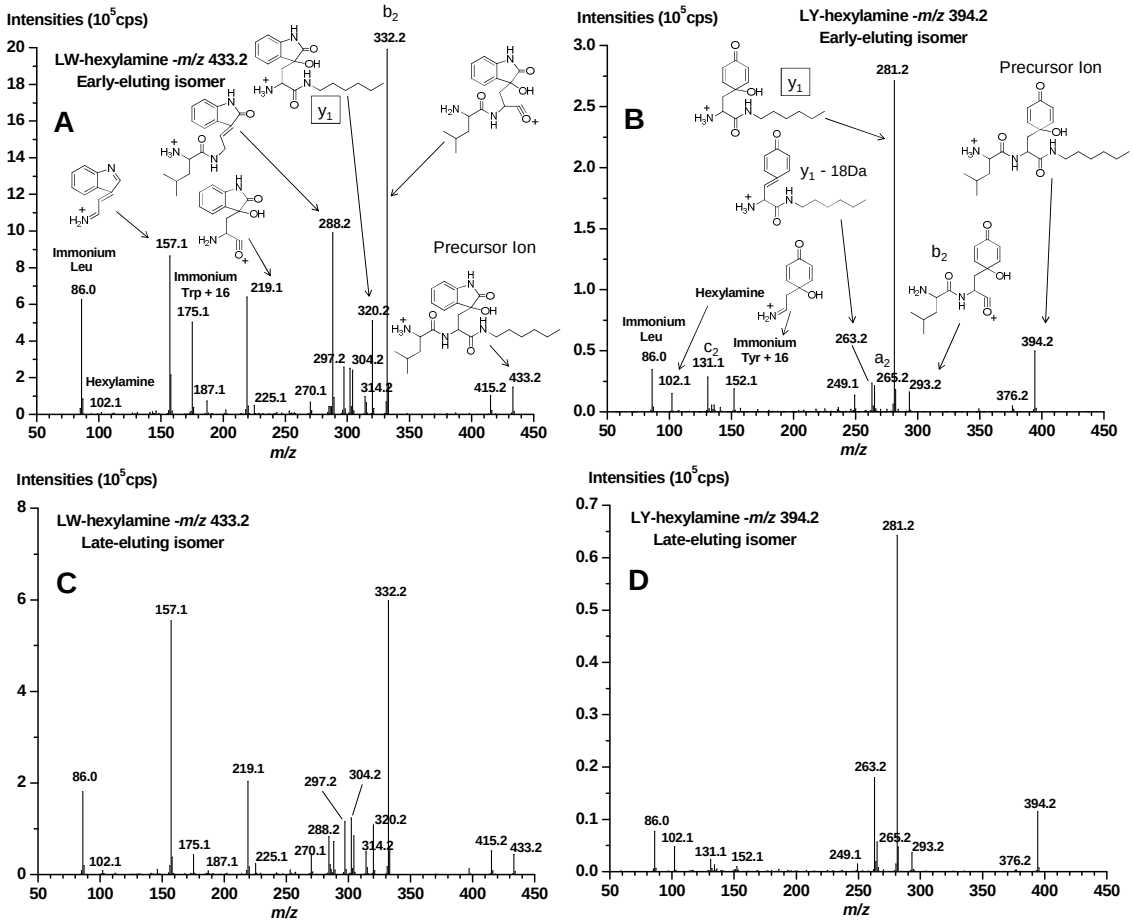


Figure 2: MS/MS spectra of hexylamine labeled spirolactones of electrochemically cleaved LYL and LWL (see Figure 1). Product ion spectra of early-eluting (A) and late-eluting (C) LW-hexylamine (m/z 433); early-eluting (B) and late-eluting (D) LY-hexylamine (m/z 394).

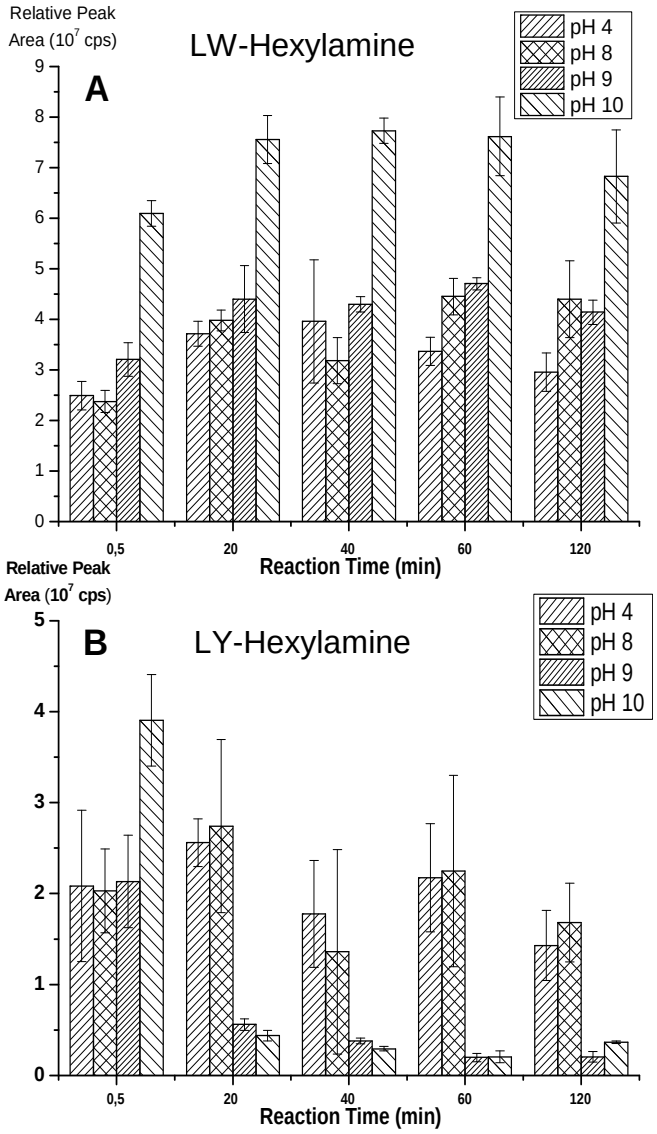


Figure 3: Effect of pH and reaction time on the formation of A) LW-hexylamine (*m/z* 433) after conjugation of the LW+14 spirolactone-containing cleavage product with hexylamine under aqueous conditions, and B) LY-hexylamine (*m/z* 394) after conjugation of the LY-2 spirolactone-containing cleavage product with hexylamine under aqueous conditions. 150 mM hexylamine (15,000-fold excess) was added to a solution of 10 µM LWL (A) or LYL (B), oxidized respectively at 700 mV and 1000 mV (vs. Pd/H_2) in $H_2O/CH_3CN/HCOOH$ (89/10/1). The pH of the solution was adjusted to 8, 9, or 10 by addition of borate buffer, and the reaction time was varied between 0.5 and 120 min at room temperature. The pH 4 sample was obtained after addition of hexylamine to the reaction mixture without pH adjustment. All experiments were performed in triplicate.

These results indicate that short reaction times of 0.5 min and pH 10 are optimal for labeling electrochemically generated Tyr and Trp cleavage products with hexylamine. These reaction times are very short compared to those needed for tagging of homoserine lactone in solution which required 6 h at 45-55ºC [11]. Our results correspond better with the 6 to 10 min reaction time at 20ºC required for successful ring opening reactions of homoserine lactones for semisynthesis of proteins and polypeptides as reported by Offord [8].

3.3 Method optimization under non-aqueous medium

The large amount of hexylamine required for efficient tagging of the spirolactone moieties under aqueous conditions (15,000-fold molar excess) limits the development of tagging reactions with more costly reagents. The labeling reaction was therefore tested with LWL under non-aqueous conditions in order to prevent hydrolysis and to reduce the excess of tagging reagent.

Several organic solvents (DMF, DMSO, CH_3CN) were tested and the best results were obtained when working in acetonitrile. Figure 4 shows the extracted ion chromatograms of the reaction products obtained after reaction of a dried solution of electrochemically oxidized LWL with a 400-fold molar excess of hexylamine in acetonitrile. The cleavage product LW+14 was converted to the LW-hexylamine labeling product (*m/z* 433) with a yield of approximately 80% relative to the remaining LW+14 spirolactone starting material. The fact that LW+14 remained after the reaction indicates that no or very little hydrolysis occurred in acetonitrile. The labeled lactone elutes as two chromatographic peaks, as observed under aqueous reaction conditions, which shows that the formation of the two isomers is independent of the solvent used for the reaction. The required amount of reagent can thus be decreased considerably under non-aqueous conditions, and the use of costly reagents such as biotin derivatives for tagging should become less prohibitive.

3.4 Precursor ion scanning of conjugated cleavage products

In CID MS/MS experiments both hexylamine-conjugated compounds generate a specific y_1 ion containing the C-terminal Tyr or Trp residue conjugated to the aliphatic hexylamine moiety (Figure 2). Labeled peptides can thus be specifically monitored in the precursor ion scanning mode. Figure 5 shows the chromatograms obtained by precursor ion scanning of the product ions at *m/z* 281.2 for LYL and *m/z* 320.2 for LWL after labeling with hexylamine under non-aqueous conditions. In both cases only the conjugated cleavage products LW-hexylamine (Figure 5A) and LY-hexylamine (Figure 5B) were observed as two peaks. Detection in the precursor ion scanning mode yields clean chromatograms which have the benefit of providing rapid and selective information on the successfully cleaved and labeled peptides. Making use of these fragments, which can be considered as reporter ions, will simplify analysis of more complex biological mixtures.

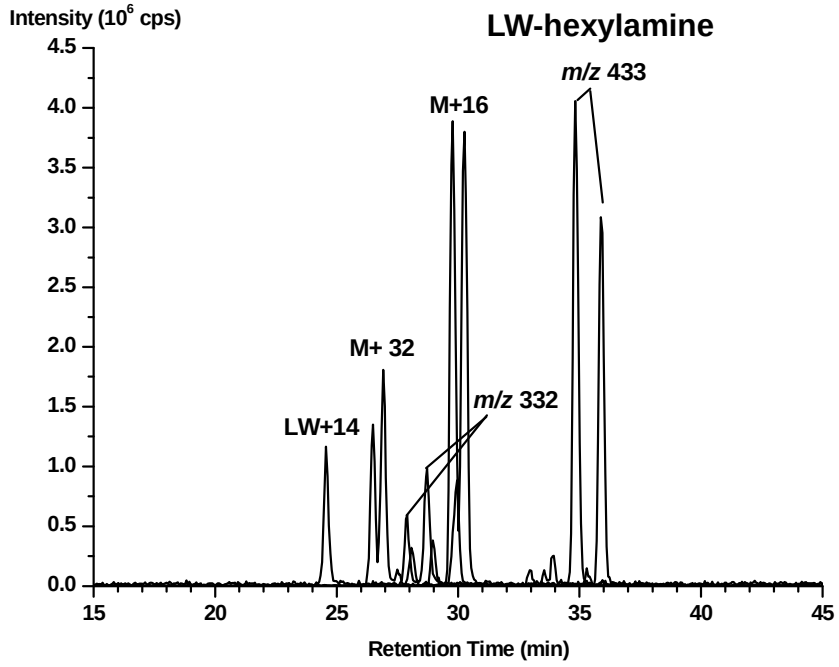


Figure 4: Combined extracted ion chromatograms of the LWL reaction products after reaction with a 400-fold molar excess of hexylamine in 50 µL acetonitrile for 20 min at room temperature. Electrochemical oxidation was performed at a peptide concentration of 10 µM in $H_2O/CH_3CN/HCOOH$ (89/10/1), a constant potential of 700 mV and a flow-rate of 50 µL/min. The solvent was evaporated prior to the tagging reaction.

4. Discussion

We show here proof-of-principle that the spirolactone moieties of the C-terminal residues formed by the electrochemically-induced oxidation and cleavage of the peptide bond next to Tyr and Trp undergo a lactone ring opening reaction in the presence of an excess of a primary amine under basic conditions. This constitutes the first chemical labeling approach of electrochemically cleaved peptides by taking advantage of the common C-terminal spirolactone. Addition of hexylamine to the reaction mixture after oxidation of the tripeptides LYL and LWL yielded the expected ring opening amidation reaction leading in both cases to the formation of labeled peptides as confirmed by LC-MS/MS analysis. Both labeled cleavage products undergo a strong chromatographic shift to longer retention times caused by the addition of the aliphatic hexyl chain (Figure 1). The strong retention time shift may, for example, be utilized in the COFRADIC workflow developed by Gevaert, Vandekerckhove and coworkers [12,13], where fractional diagonal chromatography is performed prior to, and after, chemical labeling. Combining our electrochemical cleavage-tagging reaction with diagonal chromatography may facilitate the detection of electrochemically cleaved peptides in more complex samples.

Another characteristic feature arising from this labeling method is that both hexylamine labeled compounds yield a characteristic y_1 ion after CID MS/MS that contains the C-terminal Tyr or Trp residue covalently bonded to the aliphatic hexylamine moiety. This opens the possibility to use these fragments as reporter ions for precursor ion scanning LC-MS/MS to detect the labeled products in complex reaction mixtures. Although we used a simple labeling reagent to study the feasibility of this (electro)chemical labeling workflow, it is conceivable to use labeling reagents that contain elements inducing mass defects (e.g. halogens) which would allow the unambiguous detection of tagged peptides with mass spectrometers having high-resolution and high-accuracy fragment ion selection (e.g. quadrupole TOF) [14]. Such an approach would further enhance the specificity of the precursor ion scanning approach when working with complex biological matrices. Labeling reagents with functional groups such as biotin may be introduced to enrich the labeled

peptides from complex mixtures before LC-MS analysis, as has been shown for homoserine lactone-containing peptides [11].

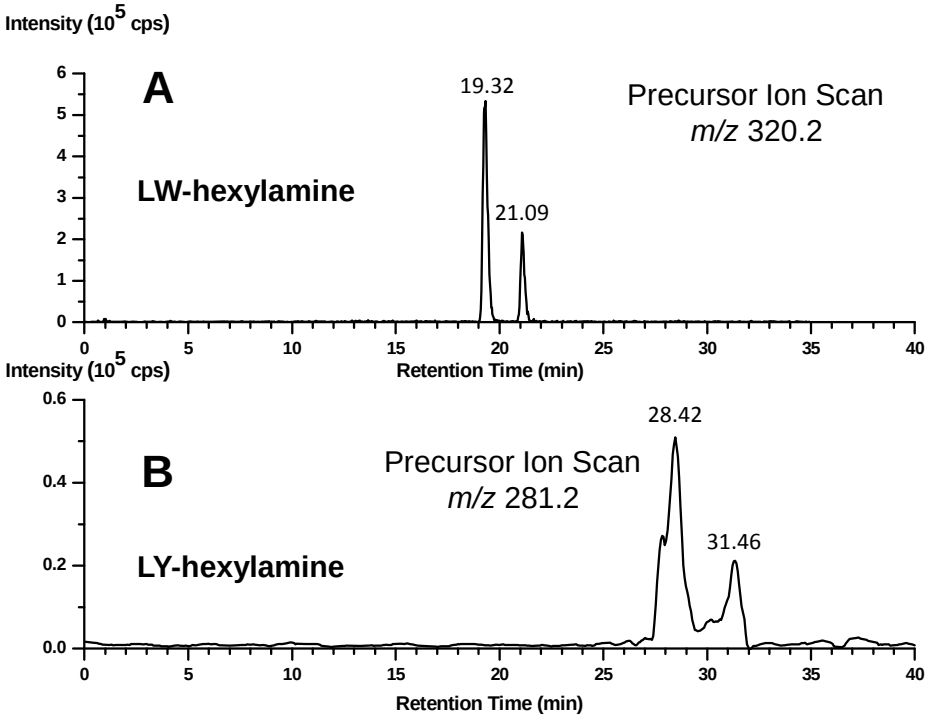


Figure 5: Precursor ion chromatogram after electrochemical oxidation and chemical labeling with a 400-fold molar excess of hexylamine under non-aqueous conditions in 50 µL acetonitrile of A) 10 µM LWL (*m/z* 320.2) and B) 10 µM LYL (*m/z* 281.2). Electrochemical oxidation was performed at a peptide concentration of 10 µM in $H_2O/CH_3CN/HCOOH$ (89/10/1), a constant potential of 700 mV for LWL and 1000mV for LYL, and a flow-rate of 50 µL/min. The solvent was evaporated prior to the tagging reaction.

5. Conclusion

The successful chemical labeling of products obtained by electrochemical cleavage of Tyr- and Trp-containing tripeptides shows the feasibility of using this approach to tag the spirolactone moieties with a primary amine. It opens the possibility of developing new strategies that specifically target cleaved peptides among a potentially complex mixture of

oxidation products, which is currently a major disadvantage of electrochemical protein digestion.

Other primary amine reagents containing specific isotopic patterns for quantitation purposes, and/or the incorporation of tags for affinity enrichment are future options to increase the applicability of the current method for the extraction/enrichment of electrochemically cleaved peptides and proteins as part of a proteomic workflow. Finally, cleavage C-terminal to Tyr and Trp introduces a specificity that is currently not accessible through enzymatic methods.

6. References

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Chapter 5

Boron-Doped Diamond Electrodes for the Electrochemical Oxidation & Cleavage of Peptides

Julien Roeser, Niels F.A. Alting, Hjalmar P. Permentier, Andries P. Bruins, Rainer Bischoff

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Submitted

Abstract

Electrochemical oxidation of peptides and proteins is traditionally performed on carbon-based electrodes. Adsorption caused by the affinity of hydrophobic and aromatic amino acids toward these surfaces leads to electrode fouling. We compared the performance of boron-doped diamond (BDD) and glassy carbon (GC) electrodes for the electrochemical oxidation and cleavage of peptides. An optimal working potential of 2000 mV was chosen to ensure oxidation of peptides on BDD by electron transfer processes only. Oxidation by electrogenerated OH radicals took place above 2500 mV on BDD, which is undesirable if cleavage of a peptide is to be achieved. BDD showed improved cleavage yield and reduced adsorption for a set of small peptides, some of which had been previously shown to undergo electrochemical cleavage C-terminal to tyrosine (Tyr) and tryptophan (Trp) on porous carbon electrodes. Repeated oxidation with BDD electrodes resulted in progressively lower conversion yields due to a change in surface termination. Cathodic pre-treatment of BDD at a negative potential in an acidic environment successfully regenerated the electrode surface and allowed for repeatable reactions over extended periods of time. BDD electrodes are a promising alternative to GC electrodes in terms of reduced adsorption and fouling and the possibility to regenerate them for consistent high-yield electrochemical cleavage of peptides. The fact that OH-radicals can be produced by anodic oxidation of water at elevated positive potentials is an additional advantage as they allow another set of oxidative reactions in analogy to the Fenton reaction, thus widening the scope of electrochemistry in protein and peptide chemistry and analytics.

1. Introduction

Carbon-based electrodes are widely used in electrochemistry and are often preferred to noble metal electrodes for oxidation of organic and biological molecules [1]. The main benefits of such materials are their electrocatalytic activity for a variety of redox reactions, their wide potential window, low cost and the possibility of preparing porous materials with large surface areas (porous graphite carbon). Graphite and glassy carbon electrodes are, however, also known to suffer from surface fouling and from oxide formation by reaction with oxygen and water. These surface reactions can have a significant effect on adsorption, electron-transfer kinetics and electrocatalysis [2].

The introduction of diamond-based materials as electrodes in the late 1980s by Pleskov *et al.* [3] was a major advance in electrochemistry. Natural diamond is an electrically insulating material that cannot be used as an electrode but the introduction of impurities within the sp^3-hybridized tetrahedral lattice of diamond makes it conductive. Boron is by far the most widely used dopant and yields p-type semiconductors or materials with metal-like electronic properties depending on the doping levels [4]. Boron-doped diamond (BDD) thin-films are mostly prepared by chemical vapor deposition (CVD) on silicon or metallic substrates under a hydrogen atmosphere yielding H-terminated surfaces. Preparation techniques, characterization, surface modifications, and electrochemical properties of BDD electrodes have been reviewed elsewhere [1,2,4-8].

BDD electrodes have become attractive materials for electrochemical applications in place of glassy carbon or graphite due to their wider potential window, low background currents, higher chemical inertness, high thermal conductivity, and high mechanical stability. The high overpotential for both oxygen and hydrogen evolution are responsible for the wide potential window (the widest so far measured in aqueous electrolytes) and allows conversion of molecules with high oxidation and reduction potentials. Chemical inertness and low background currents stem from the C-sp^3 hybridization of diamond which has a low capacitance and prevents surface oxide formation [1]. Owing to these properties, BDD is a material well-known for its low adsorption, resistance to (bio)fouling, excellent response

stability, and high current signal-to-noise ratio which made it popular for a wide range of electroanalytical applications for both inorganic and organic compounds [4,8].

Electrochemical properties and performance of BDD electrodes depend on several factors such as doping levels, non-diamond impurities (C-sp^2 carbon) and surface termination (hydrogen or oxygen). The latter has a great influence on charge transfer rates at the electrode surface which decrease upon the gradual replacement of superficial hydrogen by oxygen-terminated sites during repeated anodic oxidation or prolonged exposure to ambient air [9,10]. BDD electrode pre-treatment methods at high anodic or cathodic potentials in an acidic environment were shown to allow tuning of surface termination states in order to study reaction kinetics [10]. Cathodic pre-treatment may improve the voltammetric response of analytes [11] or can be used for the regeneration of an electrode surface that was passivated due to analyte adsorption on an O-terminated surface, as has been shown in the context of the electrochemical oxidation of proteins [12].

Anodic hydroxyl radical production by oxidation of water can occur very effectively on BDD electrodes due to the high overpotential for oxygen evolution. Hydroxyl radical generation at BDD electrodes is very specific for this material and is widely used for water treatment and destruction of organic or inorganic pollutants, which is by far the most investigated application area for BDD electrodes. Formation of hydroxyl radicals and even methoxy radicals has also been applied to other fields such as protein footprinting [13] and organic synthesis [14-16].

Electrochemical oxidation of peptides and proteins has been shown to yield specific cleavage of the peptide bonds C-terminal to Tyr and Trp residues and holds promise to become an instrumental alternative to chemical and enzymatic protein cleavage [17-19]. Electrochemical cleavage has so far mainly been carried out on purely carbon-based materials. Adsorption and fouling of carbon electrodes are aggravating issues when working with large (bio)molecules and impair repeatability and reproducibility, or even prevent oxidation to occur at all [18]. Moreover, Tyr dimer formation by crosslinking reactions [20,21] occurs on carbon-based materials [22] due to strong affinity and adsorption of the phenolic ring to the electrode surface [23]. Such drawbacks contribute to the limited

cleavage yields. BDD is thus a promising electrode material for electrochemical oxidation of peptides and proteins, owing to its limited adsorption. Studies involving a single amino acid or amino acid mixtures have shown improved performance for electrochemical detection in terms of lower adsorption and improved electrode stability, although in some cases fouling and electrode passivation have been observed when working with concentrated solutions in the millimolar range [24-30]. BDD electrodes have also been investigated for the electrochemical detection of peptides and proteins (both metallo- and non-metal-containing proteins) [12,31-38] and for studies on resistance to protein fouling [39-42].

We report here the first comparison and evaluation of the performance of BDD versus GC electrodes for the electrochemical oxidation and cleavage of peptides. Products were monitored by LC-MS which provided detailed information about the reactions occurring at the electrode surfaces. The potential regions of the two different oxidation mechanisms occurring on BDD electrodes, i.e. direct electron transfer processes and hydroxyl radical formation, were investigated as well as methods to regenerate the electrode surface after performance loss upon prolonged oxidation experiments.

2. Materials and Methods

2.1 Chemicals

The tripeptides LYL and LFL were obtained from Research Plus Inc. (Barnegat, NJ, USA). Angiotensin I (DRVYIHPFHL), Adrenocorticotropic hormone (ACTH) 1-10 (SYSMEHFRWG) and formic acid (HCOOH) were purchased form Sigma-Aldrich (Steinheim, Germany). Water was purified by an Arium Ultrapure water system (conductivity 18.2 MΩ.cm, Sartorius Stedim Biotech, Göttingen, Germany). HPLC supra gradient acetonitrile was purchased from Merck (Darmstadt, Germany).

2.2 Electrochemical oxidation of peptides

Stock solutions of LYL, LFL, Angiotensin I and ACTH 1-10 were prepared at a concentration of 1 mM in 89/10/1 (v/v/v) ultra-pure water/acetonitrile/formic acid and diluted to a final concentration of 5 µM prior to oxidation.

The tripeptide solutions were oxidized with a Flexcell thin-layer cell (Antec Leyden, Leiden, The Netherlands) with Magic Diamond (BDD) and glassy carbon (GC) working electrodes (8 mm diameter, surface area of 50.3 mm^2), and a palladium (Pd/H_2) reference electrode (Hy-REF). Prior to use, BDD electrodes were washed with methanol and ultra-pure water. Cathodic pre-treatment of BDD electrodes was performed at a constant potential of -3000 mV for 1 h in the presence of 0.5 M sulfuric acid when significant performance decrease of the electrode was detected. GC electrodes were polished using diamond spray (1 µm particles) and subsequently rinsed with ultra-pure water.

The potentials were controlled with a home-made potentiostat controlled by a MacLab system (ADInstruments, Castle Hill, NSW, Australia) and EChem software (eDAQ, Denistone East, NSW, Australia).

2.3 Liquid Chromatography - Mass Spectrometry (LC-MS)

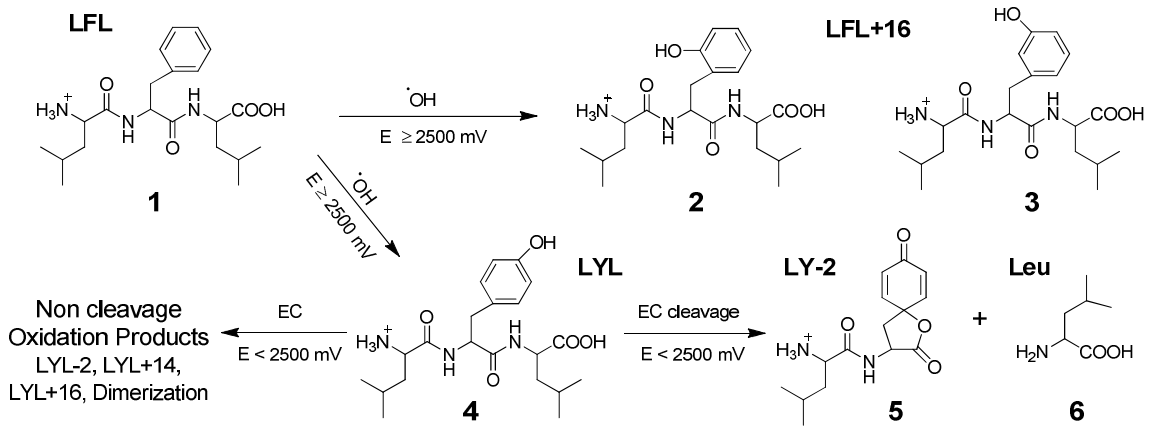
Liquid chromatography was performed on an Ultimate plus system (Dionex-LC Packings, Amsterdam, The Netherlands) equipped with an Ultimate gradient pump and Famos well plate Microautosampler. A Vydac RP-C_{18} column (150 mm × 1 mm i.d., 5 µm particles, 300 Å pore size, Grace Vydac) was used for chromatographic separation at a flow rate of 50 µL/min. Mobile phase A consisted of ultra-pure water with 0.1% formic acid. Mobile phase B was acetonitrile with 0.1% formic acid.

For analysis of the peptide-derived reaction products, 50 µL injections were performed, and separation was achieved with a gradient of B (5-50% at 1 %/min). The column was directly coupled to an API365 triple quadrupole mass spectrometer (AB-Sciex, Concord, Ontario, Canada) upgraded to EP10+ (Ionics, Bolton, Ontario, Canada) and equipped with a TurboIonSpray source for product detection in the positive ion mode.

3. Results and Discussions

3.1 Direct oxidation vs. electrochemical hydroxyl radical formation

The high overpotential for both oxygen and hydrogen evolution on BDD electrodes allows efficient anodic oxidation of water to hydroxyl radicals. Generation of hydroxyl radicals on BDD electrodes takes place at elevated potentials which can vary in our experience from one electrode to another depending on the physical, chemical and electronic properties of the material. The tripeptide LFL (**1**) (Scheme 1) was used to determine the potential limit at which hydroxyl radicals are generated in our system, since the aromatic ring of phenylalanine (Phe) cannot undergo direct oxidation by electron transfer reactions, but is a target for hydroxylation by reactive oxygen species [5].



Scheme 1: Hydroxyl-radical-induced oxidation of the tripeptide LFL on BDD electrodes. Para-hydroxylation of LFL yields the tripeptide LYL which can undergo further direct electron transfer reactions including cleavage of the peptide bond C-terminal to Tyr.

Figures 1A and 1B compare the base peak chromatograms obtained after oxidation of LFL (**1**) at a BDD electrode at 2000 mV and 2500 mV vs. Pd/H_2. The formation of hydroxyl radicals by oxidation of water occurred at 2500 mV as indicated by the presence of LFL oxidation products eluting prior to the unoxidized tripeptide in LC-MS (Figure 1B).

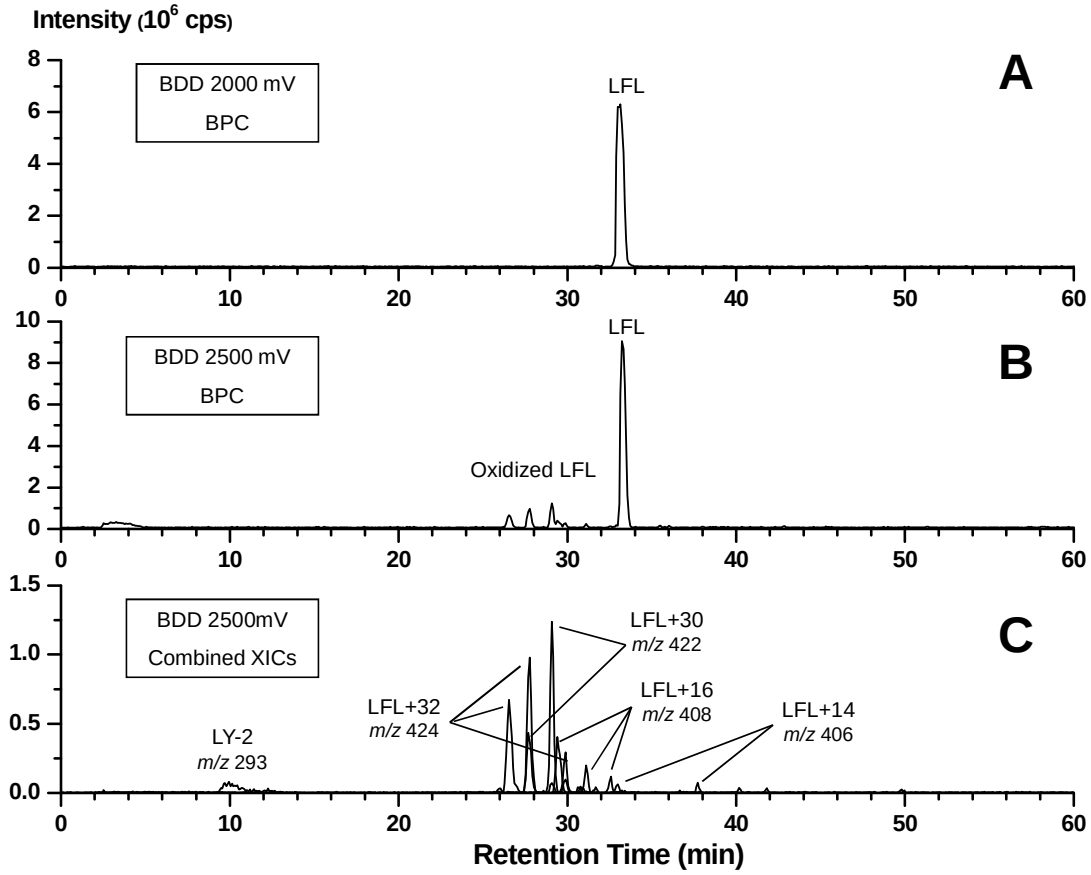


Figure 1: LC-MS chromatograms of reaction products obtained after oxidation of 5 µM LFL in $H_2O/CH_3CN/HCOOH$ (89/10/1) at a flow-rate of 5 µL/min on a BDD electrode. Base-peak chromatograms (BPC) obtained after oxidation at (A) 2000 mV and (B) 2500 mV. (C) Extracted ion chromatograms (XICs) of the oxidation products obtained at 2500 mV.

The combined extracted ion chromatograms in Figure 1C illustrate in more detail the range of products obtained for LFL (**1**) at 2500 mV. The presence of products with a mass increase of 16 Da (*m/*z 408) compared to LFL (*m/*z 392) indicate the formation of hydroxylated LFL that occurs via hydroxyl radical attack. Despite their broad range of reactivity, hydroxyl radicals preferably target oxidation-sensitive amino acid side chains (i.e. Cys, Met, Trp, Tyr, Phe and His) within peptides and proteins [5,43]. The presence of 3 peaks with a mass increment of 16 Da suggests that oxidation in the ortho-, meta- and para-

positions of the aromatic ring occurred, yielding hydroxylated LFL (**2**), (**3**) and (**4**), respectively (Scheme 1). The high peak intensities of LFL+32 Da products, also eluting as 3 chromatographic peaks, show that a second hydroxylation step is favored under these conditions. This secondary oxidation step can occur via two different mechanisms: a second hydroxyl radical attack or direct electrochemical oxidation of hydroxylated LFL. Indeed, hydroxylation of Phe in the para-position leads to the formation of Tyr and in this case the formation of the tripeptide LYL (**4**), which can react further by direct electron transfer reactions. As described in previous work [19] and shown in Scheme 1, LYL (**4**) can undergo dehydrogenation, hydroxylation or a combination of those reactions yielding products with a mass decrease of 2 Da or a mass increase of 16 Da and 14 Da, respectively. These products are all observed in Figure 1C and are labeled LFL+14, LFL+32 and LFL+30, respectively. Finally, cleavage of the peptide bond next to Tyr by direct electrochemical oxidation followed by a secondary hydrolysis step [19] yielding the dipeptide LY-2 (**5**) occurs as well as shown by the presence of the early eluting *m/z* 293 product (Figure 1C).

Oxidation products of LFL were absent at 2000 mV (Figure 1A). Therefore, anodic oxidation of water leading to the formation of hydroxyl radicals did not occur at 2000 mV making this potential suitable when only direct electrochemical oxidation reactions are desired, such as the electrochemical peptide cleavage C-terminal to Tyr or Trp.

3.2 Cathodic pre-treatment of BDD electrodes

The surface termination of BDD affects the electrochemical behavior of the electrode. Charge transfer is enhanced when working with hydrogen-terminated surfaces. Oxygen-terminated sites are formed during oxidation at positive potential in the presence of oxygen. Reversion to an H-terminated surface is possible by cathodic pre-treatment at high negative potentials in an acidic environment [10].

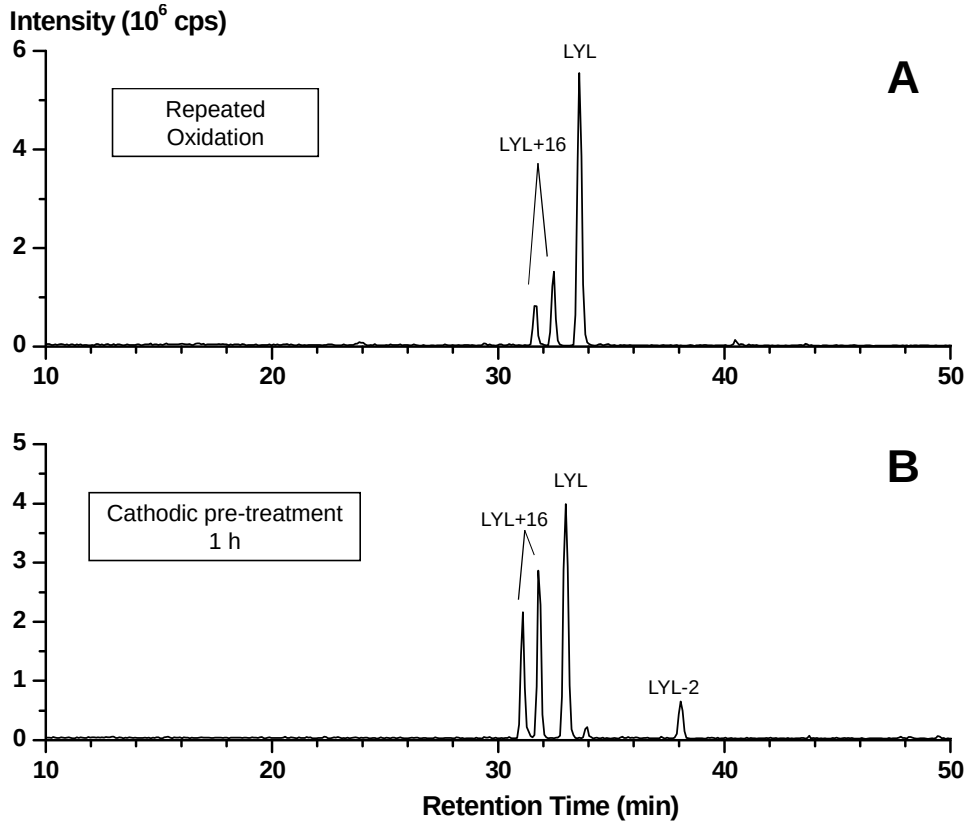


Figure 2: Influence of cathodic pre-treatment on the performance of a BDD electrode for the oxidation of 5 µM LYL in $H_2O/CH_3CN/HCOOH$ (89/10/1) at a flow-rate of 5 µL/min at 2000 mV. Base peak chromatograms of the reaction products (A) prior to and (B) after cathodic pre-treatment at a constant potential of -3000 mV for 1 h in the presence of 0.5 M sulfuric acid.

Deterioration of BDD-electrode performance was observed after repeated peptide oxidation over a period of several days by a progressive decrease of oxidation yields to 20-30%. Figure 2 illustrates the conversion of the tripeptide LYL prior to and after 1 h of cathodic pre-treatment. Electrode pre-treatment was performed by applying a constant potential of -3000 mV in the presence of 0.5 M sulfuric acid and resulted in oxidation yields of 60-70%, which are comparable to the values observed originally. This is attributed to the replacement of O-terminated by H-terminated sites. Cathodic pre-treatment has to be carried out regularly in order to ensure reliable and reproducible results. No significant drop in performance was observed during one day of peptide conversions indicating that daily regeneration the electrode is sufficient for reproducible results.

3.3. Peptide cleavage on a thin-layer GC electrode

For comparison with the BDD electrode, electrochemical oxidation was performed with a GC electrode of identical size (8 mm diameter). The tripeptide LYL was used to determine the optimal potential for maximum conversion. All experiments were performed at 5 µM peptide concentration with flow rates in the low µL/min range. Oxidation of LYL started at 1000 mV vs. Pd/H_2 but with a low oxidation yield. Maximal conversion of 30-40% was reached at a potential of 1500 mV (Figure 3A). The range of oxidation and cleavage products detected on the GC electrode in a thin-layer cell is detailed in Figure 3B and is in accordance with the range of products obtained in previous work performed with porous graphite electrodes (ESA 5021 cell) [19]. However, the amount of products was much higher in the coulometric ESA cell due to the much larger surface area of the porous graphite electrode (more than 50 times the area of the flat GC electrode). Dimers formed by Tyr cross-linking reactions, are the most abundant oxidation products of the tripeptide LYL on GC suggesting strong adsorption of the peptide to the working electrode surface. Dimerization of, and cross-linking within Tyr-containing peptides and proteins [44,45] readily occurs at the surface of carbon electrodes due to strong adsorption of the phenolic ring [23]. This constitutes a major drawback of GC electrodes, since dimers are formed at the expense of other oxidation products, notably cleavage products.

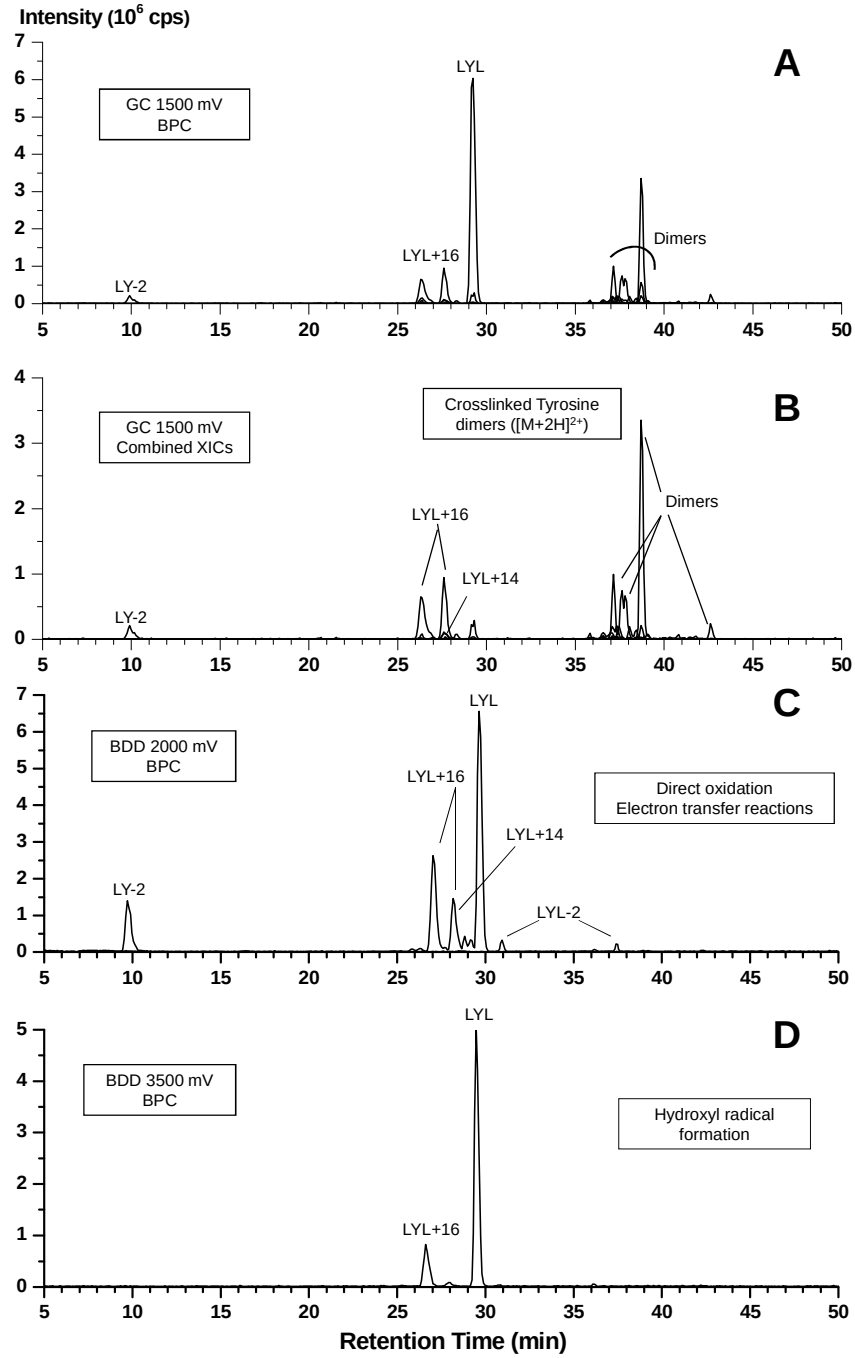


Figure 3: LC-MS chromatograms of reaction products obtained after electrochemical oxidation of 5 µM LYL in $H_2O/CH_3CN/HCOOH$ (89/10/1) at a flow-rate of 5µL/min. (A) Base peak chromatogram and (B) combined extracted ion chromatograms (XICs) of the main oxidation and cleavage products obtained on a GC electrode at 1500 mV. Base peak chromatogram obtained on a BDD electrode at (C) 2000 mV and (D) 3500 mV. Note that there is no cleavage at 3500 mV.

3.4 Peptide cleavage on a thin-layer BDD electrode

Since formation of hydroxyl radicals on the BDD electrode was observed at a potential of 2500 mV, we choose a potential of 2000 mV as the upper limit for evaluating BDD for the electrochemical oxidation and cleavage of peptides by direct electron transfer.

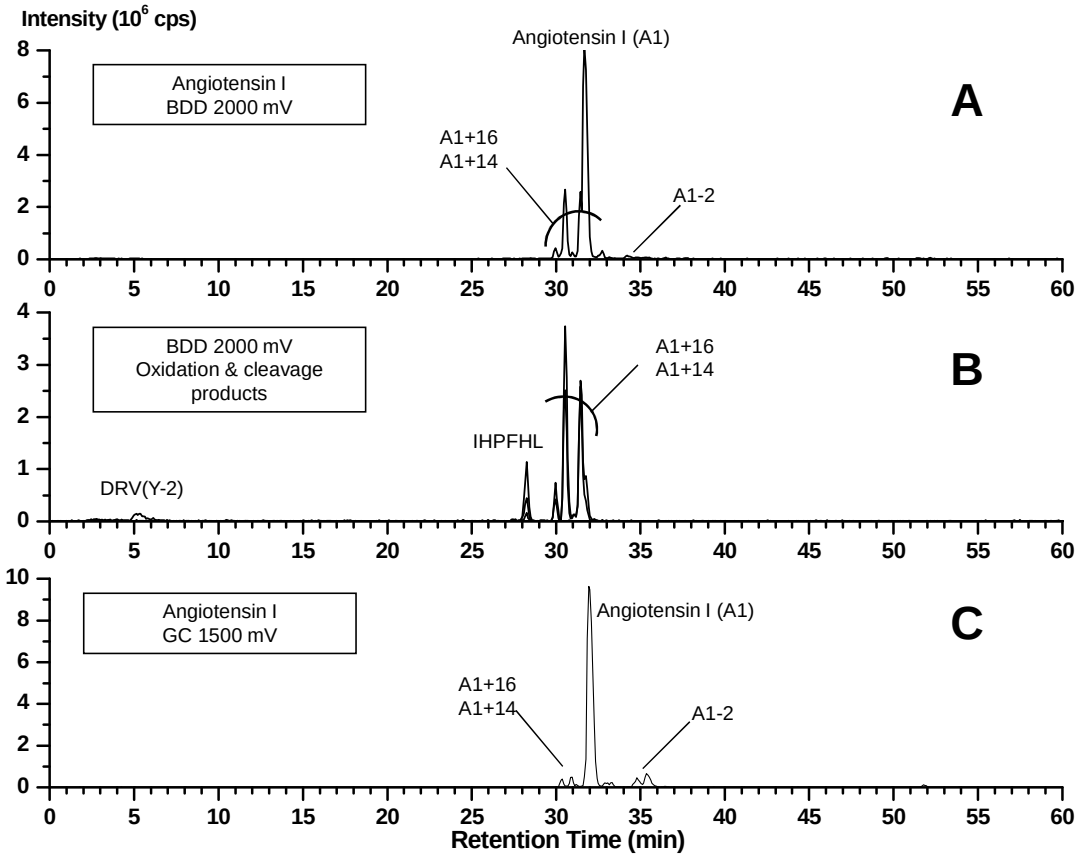


Figure 4: LC-MS chromatograms of reaction products obtained after oxidation of 5 µM Angiotensin I (DRVYIHPFHL) in $H_2O/CH_3CN/HCOOH$ (89/10/1) at a flow-rate of 5 µL/min at (A) 2000 mV on BDD and (C) 1500 mV on GC electrodes. Panel B shows the extracted ion chromatograms (XICs) of the main oxidation and cleavage products obtained on the BDD electrode at 2000 mV.

Oxidation of LYL on BDD was initiated at 1300 mV with a conversion of around 5% and no detectable cleavage products. Both oxidation and cleavage yields increased with higher potentials and maximum conversion of 50% was obtained at 2000 mV. The cleavage and non-cleavage oxidation products are presented in Figure 3C and correspond closely to

those obtained on a porous graphite electrode as previously described [17,19]. Since complete conversion can be reached with peptide concentrations of up to 50 µM on the porous graphite electrode, BDD electrodes with larger surface areas are needed to reach similar conversion levels.

Two main differences were observed between BDD and the pure carbon electrodes. First, there is no dimer formation on BDD while this is a significant reaction pathway when working with carbon electrodes as reported above for the oxidation of LYL with a GC disk electrode and in previous work with porous graphite electrode in a coulometric cell [19]. Dimer formation likely occurs on the electrode surface due to the high local concentration of adsorbed LYL. The observation that LYL does not dimerize even at high positive potentials indicates that adsorption to BDD electrodes is much lower. We therefore use the extent of Tyr-mediated dimer formation as an indirect measure of peptide adsorption. Second, when potentials greater than 2000 mV were applied, a progressive decrease of the cleavage yield with a concomitant increase of the M+16 hydroxylated products was observed (see Figure 3D for the reaction products observed at 3500 mV). This observation confirms the oxidation pathway by reaction with hydroxyl radicals at very positive potentials on the BDD electrode, and shows that this goes at the expense of cleavage product formation.

3.5 Electrochemical oxidation of larger peptides

Two decapeptides, Angiotensin I and Adrenocorticotropic hormone (ACTH) 1-10, were analyzed to compare BDD and GC electrodes with larger peptides in view of the adsorption issues encountered previously on porous graphite electrodes [17,18].

Angiotensin I (DRVYIHPFHL) contains a single Tyr residue that is amenable to direct electrochemical oxidation. Earlier experiments with porous graphite electrodes [17] showed that it is possible to cleave this peptide C-terminal to the Tyr residue but ith poor reproducibility, presumably due to adsorption of the peptide at the electrode surface. Angiotensin I was successfully oxidized at the BDD electrode in the thin layer cell with a conversion yield of 20-30% (Figure 4A), which was lower than for LYL. Lower conversion yields for larger peptides may be explained by their lower diffusion rates. While oxidation

yielded mainly non-cleavage products, listed as A1-2, A1+14, A1+16 in Figure 4A and 4B, the cleavage products DRVY-2 and IHPFHL were clearly detectable (Figure 4B). When the performance of the BDD and the GC electrodes in the thin-layer cell was compared with respect to the conversion of Angiotensin I (Figure 4C), a significantly lower yield was observed with the GC electrode. This lower efficiency may again be explained by adsorption on the surface of the GC electrode leading to a low recovery of oxidation products. Dimer formation was however not detected, possibly due to the low oxidation yields or to adsorption of the dimer on the electrode surface.

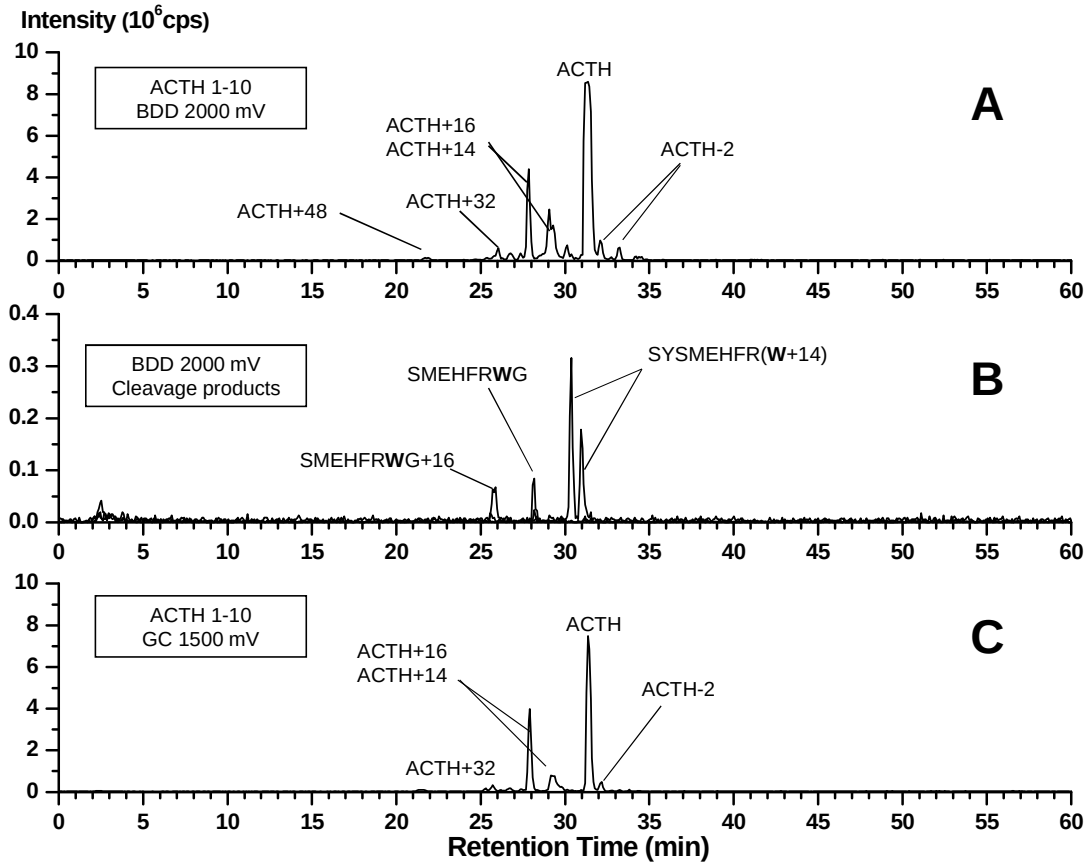


Figure 5: LC-MS chromatograms of reaction products obtained after oxidation of 5 μM ACTH 1-10 (SYSMEHFRWG) in $H_2O/CH_3CN/HCOOH$ (89/10/1) at a flow-rate of 5 μL/min at (A) 2000 mV on BDD and (C) 1500 mV on GC electrodes. (B) Extracted ion chromatograms (XICs) of the cleavage products obtained on the BDD electrode at 2000 mV.

The second peptide we evaluated was ACTH 1-10 (SYSMEHFRWG). This peptide has been tested earlier with porous graphite electrode-containing coulometric cells but no products were detected, presumably due to very strong adsorption on the porous graphite electrode [18]. It was thus of interest to determine whether lower adsorption on the BDD electrode would allow identification of oxidation or cleavage products. Another interesting feature of this peptide is that it contains 3 residues, i.e. Tyr, Met and Trp, that can be readily oxidized electrochemically by direct electron transfer reactions. This peptide is therefore useful to determine which oxidation pathways occur preferentially, and whether cleavage will occur next to both Tyr and Trp under the same conditions.

Oxidation of ACTH 1-10 at 2000 mV at the BDD working electrode showed non-cleavage oxidation products (Figure 5A) containing the expected ACTH-2, ACTH+14, ACTH+16, ACTH+32, ACTH+48 in analogy to those obtained with LYL and LWL [19]. In addition, two out of the three possible cleavage products were detected as shown in Figure 5B, namely the C-terminal peptide formed by cleavage next to Tyr (SMEHFRWG) and the N-terminal peptide formed by cleavage next to Trp (SYSMEHFRW+14). The corresponding small cleavage products (SY-2 and G) were not detected probably due to poor retention on the reversed-phase column during LC-MS analysis. The higher intensity of the Trp cleavage product suggests that this reaction is favored under these conditions. Interestingly, the Trp cleavage product eluted in two peaks suggesting the formation of isomers (Figure 5B). This has not been reported in our earlier study of Trp-containing tripeptides (performed on porous carbon electrodes) [19]. This might indicate the formation of conformational isomers that can be detected due to the higher oxidation and cleavage yields obtained on BDD. Conformational isomers have also been observed after chemical labeling of Trp-containing peptides after electrochemical cleavage [46]. Modification of oxidizable amino acids present in the cleavage products of ACTH 1-10 did not always take place. S-oxidation of Met within the N-terminal peptide SMEHFRW+14 was not detected. Oxidation within the C-terminal peptide obtained after cleavage next to Tyr (SMEHFRWG+16) was observed on the other hand, most likely due to hydroxylation of Trp. The thin-layer cell containing a GC electrode of identical dimensions resulted in lower

conversion and cleavage yields possibly due to peptide adsorption as indicated by dark deposits on the GC electrode (Figure 5C).

4. Conclusion

Boron-doped diamond (BDD) was investigated as electrode material in comparison to the more commonly used glassy carbon (GC) electrodes with respect to the electrochemical oxidation and cleavage of peptides. Lower adsorption of peptides on BDD was indicated by the absence of Tyr dimer formation at the electrode surface, even at elevated potentials as opposed to high amounts of Tyr dimers on GC. The maximal potential on BDD was limited to 2000 mV vs. Pd/H_2 to prevent hydroxyl radical formation occurring at 2500 mV vs. Pd/H_2. Cathodic pre-treatment of the BDD electrode was shown to be effective in regenerating the electrode surface to ensure maximal conversion rates and to obtain reliable and reproducible results even after repeated use of the electrode. Investigation of the larger decapeptides Angiotensin I and ACTH 1-10 demonstrated the benefit of BDD in comparison to GC in achieving cleavage of the peptide bond C-terminal to Tyr and Trp.

5. References

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Chapter 6

Summary and Future Perspectives

Summary

Electrochemistry combined with mass spectrometry (EC-MS) is a fairly recent technique which has the potential of modifying and characterizing molecules in an integrated analytical fashion. Electrochemistry allows fast product formation under mild, reagent-free conditions and can be tuned in terms of electrode material, cell design, applied potential and solvent conditions to serve a wide range of applications. Mass spectrometry provides sensitive detection and detailed structural information and can be directly coupled to an electrochemical cell for time-resolved analysis of potentially reactive and unstable compounds. The combination of both techniques has witnessed growing interest for a variety of applications in different research areas. The present thesis focuses on the use of EC-MS to study the electrochemical oxidation and cleavage of peptides and proteins. EC has been shown to mediate cleavage reactions C-terminal to tyrosine (Tyr) and tryptophan (Trp) within peptides and proteins. The method holds promise to develop into an integrated instrumental technique that is complementary to conventional enzymatic and chemical cleavage methods. The specific cleavage next to Tyr and Trp, which is not possible enzymatically (chymotrypsin cleaves at these residues but with low specificity), may provide a new tool for the protein chemistry community.

Chapter 1 begins with an introduction to the early developments, challenges and main applications in the field of EC-MS that have proven, or have the potential to be useful. A brief historical overview summarizes the major breakthroughs in the field from the first analysis of electrogenerated volatile compounds detected with electron impact (EI) mass spectrometry in the late 1970s to the implementation of electrochemical cells on-chip on integrated EC-MS systems that allow fast analyses with low sample volumes. Advances in the coupling of MS ionization sources with liquid sample introduction in the mid-1980s was a major contribution to the field since it allowed direct detection of non-volatile compounds and gave rise to many new opportunities in various scientific areas. We review the use of electrochemistry, in some cases inherent to electrospray ionization (ESI) sources, for analyte signal enhancement, tagging reaction purposes, simulation of drug metabolism and protein research. *In vitro* production of metabolites by EC-MS allows rapid identification and

characterization of potentially toxic metabolites in the early stage of drug development and can avoid costly and time-consuming *in vivo* methods conventionally used for drug screening. Several strategies including direct oxidation, EC-mediated generation of reactive species and electrode surface modifications with enzymes or synthetic porphyrins have been developed and led to significant advances in the perpetual effort of mimicking enzymatic metabolism reactions. In this chapter we focus on the specific cleavage of peptides and proteins as an instrumental alternative for protein digestion and the reductive cleavage of disulfide bridges. Furthermore, practical aspects arising from the hyphenation of EC and MS are addressed to emphasize technical issues that should be taken into consideration to make both techniques fully compatible.

Chapter 2 provides a review of oxidative protein labeling techniques developed in conjunction with mass-spectrometry based proteomics. The review summarizes reactions of oxidizing agents with peptides and proteins, the corresponding methodologies and instrumentation, and the major, innovative applications described in selected literature from the last decade. Two forms of oxidative labeling reactions can be distinguished: the primary (direct) oxidation of amino acids and oxidation mediated by reactive reagents. Direct oxidation reactions aim at specific functional groups in amino acids, primarily Cys, Met, Tyr and Trp, and may be followed by subsequent reaction with nucleophiles whereas reactive reagents have a broad range of reactivity and yield non-specific oxidation reactions. Methods for the production of reactive agents (reactive oxygen, nitrogen or halogen species) by chemical, photochemical, enzymatic and electrochemical means are described in detail in the first part of the review. Next, we highlight and discuss selected applications where oxidation facilitates sample preparation and purification, and those where oxidation changes physico-chemical properties with respect to chromatographic separation, ionization efficiency or mass changes for improved or differential detection of labeled peptides. Oxidative labeling of peptides and proteins expands the toolbox of biochemists and a variety of strategies for targeting amino acid side-chains have been developed, in particular for sulfur-containing and aromatic residues which play essential roles in protein function and biological recognition processes. Although the various techniques still suffer in many cases from incomplete conversion and selectivity, several applications in biological systems have

shown their usefulness. It is, for instance, to be expected that oxidative labeling will help to improve our understanding of protein-protein interactions with respect to protein function and signaling processes.

Chapter 3 presents a mechanistic study of electrochemical peptide cleavage performed on a set of Tyr- and Trp-containing tripeptides which do not suffer from adsorption issues encountered with larger peptides or proteins. Electrochemical oxidation and the subsequent chemical reactions including peptide cleavage were investigated in both on-line EC-MS and EC-LC-MS setups that provide complementary information. The present investigation is the first detailed study of electrochemical oxidation and cleavage of Trp-containing peptides. The effect of adjacent amino acids by on-line EC-MS showed that the ratios of oxidation and cleavage products are peptide-dependent and that the adjacent amino acid can influence the secondary chemical reactions occurring after the initial oxidation. Cleavage yields were generally higher for Tyr-containing peptides and up to 80% cleavage yield was obtained for GYG a peptide with minimal steric hindrance. Control of electrode potential and solvent composition were crucial to avoid unwanted reactions such as dimer formation for Tyr and an increasing number of oxygen insertions for Trp, which are detrimental to the yield of the targeted cleavage pathway. While the formation of reactive intermediates after the first oxidation step was not strongly affected when varying pH and supporting electrolyte, working under strongly acidic conditions (pH 1.9-3.1) led to optimal cleavage yields (40-80%) while little or no cleavage was observed under basic conditions. Finally, LC-MS/MS analysis gave detailed information and further insight into the range of oxidation and cleavage products.

In **chapter 4** we report a chemical labeling approach for the tagging of cleavage products generated after electrochemical oxidation of Tyr- and Trp-containing tripeptides. Electrochemically cleaved peptides have the particularity to generate C-terminal acid stable, spirolactone functional groups. Hexylamine was chosen to show the feasibility of spirolactone ring opening reactions under basic conditions. The influence of pH and reaction time was investigated and revealed that short reaction times of 30 s at high pH (pH 10) were best suited for high tagging yields of both Tyr- and Trp-containing peptides in an aqueous

environment. The large excess of reagent that was required for efficient tagging in aqueous solutions could be significantly decreased by working under non-aqueous conditions in acetonitrile, thus avoiding competing hydrolysis reactions at high pH. The structure of the successfully cleaved and tagged peptides was confirmed by CID-MS/MS. We observed two isobaric reaction products based on different chromatographic retention times with a similar fragmentation pattern suggesting the formation of conformational isomers. The precursor ion scanning mode was used to selectively detect the labeled peptides within the mixture of oxidation products by targeting a characteristic fragment of the labeled peptides. This study constitutes the first chemical labeling approach of electrochemically cleaved peptides by taking advantage of the C-terminal spirolactone. It opens the possibility of developing new strategies to specifically target cleaved peptides among potentially complex mixtures of oxidation products, which is currently a major challenge of electrochemical protein digestion.

Chapter 5 addresses the well-known adsorption issues encountered during electrochemical oxidation of peptides and proteins on the commonly used glassy carbon (GC) and porous graphite electrodes. Adsorption and fouling of carbon electrodes is a major shortcoming of electrochemical cleavage of larger peptides and proteins and impairs repeatability and reproducibility, or even prevents oxidation to occur at all. The performance of boron-doped diamond (BDD), a promising electrode material in terms of adsorption properties, has been evaluated and compared to traditional GC electrodes for electrochemical oxidation of peptides. The region of water stability was first defined by determining the potential threshold of hydroxyl radical formation, a well-known mechanism occurring on BDD electrodes. Oxidation of LFL, a peptide that is insensitive to direct oxidation by electron transfer reactions, was observed at 2500 mV (vs. Pd/H_2) indicating anodic oxidation of water and formation of hydroxyl radicals. Direct electrochemical oxidation of a set of model peptides on BDD at 2000 mV, to ensure direct electron transfer reactions only, showed lower adsorption and improved performance in terms of oxidation and cleavage yields in comparison to GC electrodes. While dimer formation due to Tyr cross-linking reactions was the most prominent side reaction on GC for the peptide LYL, suggesting strong adsorption to the electrode surface, this was not observed on BDD.

Investigation of the larger decapeptides Angiotensin I and ACTH 1-10 further demonstrated the benefit of working with BDD in comparison to GC in terms of lower adsorption leading to easily detectable specific cleavage of the peptide bond C-terminal to Tyr and Trp. Finally, cathodic pre-treatment of BDD at a negative potential in an acidic environment was shown to successfully regenerate the electrode surface to ensure maximal conversion rates and to obtain reliable and reproducible results even after repeated use of the electrode.

Future Perspectives

Electrochemical cleavage of proteins next to Tyr and Trp residues has potential for proteomics applications but still suffers from limited cleavage yields as well as issues related to adsorption to the electrode surface which affects oxidation yields and product composition. Competing non-cleavage oxidation reactions are difficult to prevent and increase the complexity of the resulting peptide mixture. Further method optimization of the chemical labeling approach of electrochemically generated cleaved peptides, as introduced in **chapter 4** of this thesis, has the potential to capture and enrich the spirolactone-containing intermediates and facilitate their detection. Derivatization with affinity tags, stable isotope labels or fluorescent dyes are promising future strategies. Chemical labeling of spirolactone with biotin affinity tags is an option for subsequent affinity purification and enrichment of the cleaved peptides with avidin or streptavidin beads. Alternatively, primary amine-containing stationary phases can be used for enrichment by binding the N-terminal cleavage products via the spirolactone functional groups. The latter approach may be combined with linking the primary amine to the stationary phase with a cleavable spacer such as a disulfide bond. Both approaches would allow reducing the complexity of the mixture by removing unoxidized or non-cleavage oxidation products and facilitate detection of electrochemically generated cleaved peptides and proteins. Other primary amine reagents of interest are stable isotope tags, which can be used for absolute and relative quantification, and fluorescent dyes for sensitive spectrophotometric detection. Development and optimization of such derivatization strategies would facilitate the application of the technique to the analysis of complex protein mixtures from cells in culture or biofluids.

The design of electrochemical cells is another area that requires further development and optimization since it significantly influences EC reaction yields. Our work presented in **chapter 5** introduces BDD electrodes to address adsorption issues encountered when oxidizing large peptides and proteins on traditional carbon electrodes. Oxidation on BDD electrodes can give higher yields, while adsorption is significantly lower than on GC. The performance of such an electrode depends on its intrinsic physical, chemical and electronic properties (e.g. doping level, presence of non-diamond impurities, crystalline form), which should be evaluated to find the appropriate material for electrochemical cleavage. Regeneration of surface termination by cathodic pre-treatments is another important aspect to control to ensure repeatability. A further important step towards the integration of EC in proteomics workflows is miniaturization to handle small sample volumes and improve efficiency. Fabrication of microfluidic chips containing three-electrode EC cells has been achieved and successfully applied to drug metabolism studies. Design of microfluidic flow-through chips with suitable working electrode materials that would oxidize and cleave peptides and proteins in a similar fashion than macroscopic EC cells is perfectly conceivable. Moreover, miniaturization offers the advantage of further system integration, which can be used to incorporate ESI needles and/or LC columns on-chip. This will allow separate 'unit-operations' on chip to perform the different tasks of a typical proteomics workflow in a spatially resolved manner. Incorporation of a reactor on-chip for post-cleavage derivatization is a realistic option and would allow a generic, fully integrated microfluidic device for proteomics application without the need for enzyme catalysts.

Electrochemical cleavage thus holds promise to advance proteomics research and protein analysis in general. It will help addressing practical problems in the areas of biomarker research but also in the field of biopharmaceuticals, a new class of highly promising pharmaceutical products that today account for more than one third of all truly novel therapeutics that are currently introduced on the market. Protein analysis in the broadest sense is crucial in many areas of society with vast economic impact ranging from the discovery of diagnostic and prognostic biomarkers for disease to the analysis of protein pharmaceuticals all the way to assessing food safety and environmental pollution. New

analytical and instrumental tools to facilitate and automate various stages of the experimental workflow are in high demand, in particular to address certain challenging classes of proteins, such as transmembrane proteins, which constitute the largest group of proteins that are currently addressed as drug targets.

Chapter 7

Nederlandse Samenvatting

Samenvatting

De combinatie van elektrochemie met massaspectrometrie (EC-MS) is een vrij recente techniek waarmee modificatie en identificatie van moleculen middels een geïntegreerde analytische benadering mogelijk is. Electrochemie maakt snelle productvorming mogelijk onder milde, reagentia-loze condities in een breed scala aan toepassingen, door de keuze van elektrodemateriaal, celontwerp, toegepaste potentiaal, en oplosmiddelcondities. Massaspectrometrie kan direct worden gekoppeld aan een elektrochemische cel waardoor gevoelige detectie en gedetailleerde structuurinformatie van reactieve en onstabiele stoffen met temporale resolutie kan worden verkregen. De combinatie van beide technieken kent een groeiende belangstelling door diverse toepassingen in verschillende vakgebieden. Dit proefschrift richt zich op het gebruik van EC-MS voor het bestuderen van elektrochemische oxidatie en splitsing van peptides en eiwitten. Er is aangetoond dat met EC, C-terminale splitsing ten opzichte van tyrosine (Tyr) en tryptofaan (Trp) in peptides en eiwitten kan plaatsvinden. Deze methode heeft de potentie om verder te worden ontwikkeld tot een geïntegreerde instrumentele techniek die complementair is aan enzymatische en chemische splitsingsmethodes. De specifieke splitsing naast Tyr en Trp, die enzymatisch niet mogelijk is (chymotrypsine knipt wel na deze aminozuren maar met lage specificiteit), kan een nieuwe, bruikbare methode zijn op het gebied van eiwitchemie.

Hoofdstuk 1 begint met een introductie van de beginjaren, de uitdagingen en belangrijkste toepassingen op EC-MS gebied met bewezen of potentiële toepasbaarheid. Een kort historisch overzicht beschrijft belangrijke doorbraken vanaf de eerste analyse van elektrochemisch gemodificeerde vluchtige stoffen, gedetecteerd met elektron impact (EI) massaspectrometrie (laat zeventiger jaren, 20e eeuw), tot de recente implementatie van elektrochemische cellen op een chip in een geïntegreerd EC-MS systeem waarmee snelle analyses met kleine monstervolumes mogelijk zijn geworden. Ontwikkelingen betreffende de koppeling van MS ionisatiebronnen met monsterintroductie in vloeibare vorm in het midden van de jaren tachtig van de 20e eeuw hebben een belangrijke bijdrage geleverd aan het vakgebied door de mogelijkheid tot directe detectie van niet-vluchtige verbindingen, en waren tevens van belang voor nieuwe ontwikkelingen in verschillende vakgebieden. We

bespreken het gebruik van EC, in sommige gevallen inherent aan electrospray-ionisatie (ESI), op het gebied van signaalversterking van het analiet, labelingsreacties, simulatie van medicijnmetabolisme, en eiwitonderzoek. *In vitro* productie van metabolieten met EC-MS maakt snelle identificatie en identificatie van potentieel toxische metabolieten in een vroeg stadium van medicijnontwikkeling mogelijk en kan het gebruik van kostbare en tijdrovende traditionele *in vivo* methodes, gebruikt voor medicijn-screening, beperken. De strategieën die hiervoor gebruikt worden zijn de volgende: directe oxidatie, Elektrochemische vorming van reactieve deeltjes en modificatie van het elektrodeoppervlak met enzymen of synthetische porfyrines die hebben geleid tot vooruitgang in de pogingen tot nabootsing van metabolisme door enzymen. In dit hoofdstuk richten we ons ook voor een belangrijk deel op de specifieke splitsing van peptides en eiwitten als een alternatief voor enzymatische of chemische eiwitdigestie en reductieve splitsing van zwavelbruggen. Tenslotte komen een aantal technische aspecten aan de orde die van belang zijn voor de koppeling van EC en MS en de volledige compatibiliteit van beide technieken.

Hoofdstuk 2 bespreekt oxidatieve eiwit-labeling technieken die zijn ontwikkeld voor op MS gebaseerde proteomics. Een opsomming van reacties met oxidatieve reagentia voor peptides en eiwitten met corresponderende methoden en instrumentatie, en de belangrijkste innovatieve toepassingen uit de literatuur van het laatste decennium worden weergegeven. Er kan onderscheid worden gemaakt tussen twee types oxidatieve labeling-reacties: een primaire (directe) oxidatie van aminozuren en oxidatie middels reactieve reagentia. Directe oxidatiereacties zijn gericht op specifieke functionele groepen van aminozuren, met name Cys, Met, Tyr en Trp, en kunnen worden gevolgd door een reactie met nucleofielen, terwijl reactieve reagentia een breed reactiviteitsgebied hebben wat resulteert in niet-specifieke oxidatiereacties. Het eerste deel van de bespreking beschrijft in detail welke methodes voorhanden zijn voor de productie van reactieve agentia (stoffen die reactieve zuurstof, stikstof of halogenen bevatten) gebaseerd op chemische, fotochemische, enzymatische en elektrochemische reacties. Vervolgens worden verschillende toepassingen besproken waarbij oxidatie wordt gebruikt bij monstervoorbewerking en opzuivering, naast toepassingen waarbij de verandering van fysisch-chemische eigenschappen als gevolg van oxidatie wordt gebruikt voor chromatografische scheiding, ionisatie-efficiëntie of

massaverandering voor verbeterde of differentiële detectie van gelabelde peptides. Oxidatieve labeling van peptides en eiwitten vergroot de toolbox voor biochemici en een verscheidenheid aan strategieën voor het labelen van aminozuurzijketens zijn ontwikkeld, in het bijzonder voor zwavelbevattende en aromatische aminozuren. Hoewel verschillende technieken nog last hebben van incomplete omzetting en selectiviteit zijn er verschillende nuttige toepassingen in biologische systemen beschreven. Het ligt in de verwachting dat oxidatieve labeling zal bijdragen aan onze kennis van eiwit-eiwit interacties op het gebied van eiwitfunctie en signaaltransductieprocessen.

Hoofdstuk 3 geeft een studie weer over het werkingsmechanisme van elektrochemische peptidesplitsing, uitgevoerd op een set van Tyr- en Trp-bevattende tri-peptiden. Deze hebben geen last van adsorptie-effecten zoals bij grotere peptiden en eiwitten worden waargenomen. Elektrochemische oxidatie en de daaropvolgende chemische reacties, waaronder peptidesplitsing, zijn onderzocht in zowel on-line EC-MS als EC-LC-MS opstellingen waaruit complementaire informatie wordt verkregen. Dit onderzoek is de eerste gedetailleerde studie van elektrochemische oxidatie en splitsing van Trp-bevattende peptiden. On-line EC-MS laat het effect van het aangrenzende aminozuur zien op de verhoudingen van oxidatie- en splitsingproducten. Deze zijn peptide-afhankelijk en het aangrenzende aminozuur kan van invloed zijn op secundaire chemische reacties die optreden na de eerste oxidatie. Splitsingsefficiënties zijn in het algemeen groter bij Tyr-bevattende peptiden. Tot wel 80% opbrengst werd verkregen met het peptide GYG, een peptide met minimale sterische hindering. Controle over de elektrodepotentiaal en oplosmiddelsamenstelling waren cruciaal om ongewenste reacties te voorkomen, zoals dimeervorming van Tyr en een toenemend aantal inserties van zuurstofatomen in Trp. Deze zijn ongunstig voor de peptideopbrengst van de beoogde splitsingsreactie. De vorming van reactieve intermediairen na de eerste oxidatiestap werd nauwelijks beïnvloed wanneer de pH en elektrodepotentiaal werden gevarieerd. Optimale splitsingsopbrengsten (40-80%) werden verkregen bij zure condities (pH 1.9-3.1) terwijl weinig tot geen splitsing werd waargenomen bij basische condities. Ten slotte leverde LC-MS/MS analyse gedetailleerde informatie en diepgaander inzicht op over de variatie in oxidatie- en splitsingsproducten.

In **hoofdstuk 4** rapporteren we een chemische labelingsstrategie voor het labelen van splitsingsproducten, gegenereerd na elektrochemische oxidatie van Tyr- en Trp-bevattende tripeptiden. Elektrochemisch gesplitste peptiden hebben de bijzonderheid om C-terminale, zuur-stabiele, spirolacton functionele groepen te vormen. Hexylamine werd gekozen om de haalbaarheid van spirolacton-ringopeningsreacties onder basische condities aan te tonen. De invloed van de pH en de reactietijd is onderzocht en daaruit bleek dat korte reactietijden van 30 seconden bij hoge pH (pH 10) het meest geschikt zijn om hoge labelingsopbrengsten voor zowel Tyr- als Trp-bevattende peptiden te verkrijgen in een waterig milieu. Een grote overmaat aan reagens die nodig was om een efficiënte labeling te verkrijgen in waterig milieu kon significant worden verminderd door te werken in niet-waterige milieu (acetonitril). Zo werd voorkomen dat competitieve hydrolysereacties bij hoge pH optreden. De structuur van de succesvol gesplitste en gelabelde peptiden werd bevestigd door CID-MS/MS. Wij namen isobare reactieproducten waar op basis van verschillen in chromatografische retentietijden, maar met gelijke fragmentatiepatronen, wijzend op de vorming van conformationele isomeren. De precursor ion scanning modus werd gebruikt om selectief gelabelde peptiden te detecteren in een mengsel van oxidatieproducten, door te richten op karakteristieke fragmenten van de gelabelde peptiden. Deze studie levert de eerste labelingsstrategie voor elektrochemisch gesplitste peptiden door het benutten van het C-terminale spirolacton. Het opent mogelijkheden voor de ontwikkeling van nieuwe strategieën om gesplitste peptiden specifiek aan te tonen in potentieel complexe mengsels van oxidatieproducten, hetgeen op dit moment een enorme uitdaging is bij elektrochemische eiwitsplitsing.

Hoofdstuk 5 behandelt het welbekende adsorptieprobleem, dat optreedt bij elektrochemische oxidatie van peptiden en eiwitten op de gebruikelijke glassy carbon (GC) en poreuze-grafietelektroden. Adsorptie en vervuiling van koolstofelektroden is een grote tekortkoming van elektrochemische splitsing van grotere peptiden en eiwitten. Het belemmert de herhaalbaarheid en reproduceerbaarheid en het kan zelfs voorkomen dat oxidatie volledig wordt voorkomen. De resultaten van met borium verrijkte diamant (BDD), een veelbelovend elektrodemateriaal wat betreft adsorptieeigenschappen, is geëvalueerd en vergeleken met de traditionele GC electroden voor elektrochemische oxidaties van peptiden.

De elektrochemische stabiliteit van water werd eerst vastgesteld d.m.v. bepaling van de drempelwaarde voor de potentiaal voor de vorming van hydroxylradicalen, een bekend mechanisme dat voorkomt bij BDD-electroden. Oxidatie van LFL, een peptide dat ongevoelig is voor directe oxidatie door elektronenoverdrachtreacties, werd waargenomen bij 2500 mV (versus Pd/H_2), hetgeen een indicatie is voor anodische oxidatie van water en de vorming van hydroxylradicalen. Directe elektrochemische oxidatie van een set model peptiden op een BDD-electrode bij 2000 mV, om zeker te zijn van alleen directe elektronenoverdrachtreacties, lieten lagere adsorptie en verbeterde prestaties zien voor oxidatie- en splitsingsopbrengsten, in vergelijking met GC electroden. Dimeervorming door Tyr crosslinking-reacties is de meest voorkomende nevenreactie op GC voor peptide LYL. Dit veronderstelt sterke adsorptie aan het elektrodeoppervlak, hetgeen niet werd waargenomen op BDD. Onderzoek naar de grotere decapeptiden Angiotensine 1 en ACTH 1-10 liet verder het voordeel zien van het werken met BDD. In vergelijking met GC waren de specifieke splitsingsproducten van de peptidebinding C-terminaal van Tyr en Trp gemakkelijker te detecteren. Tenslotte gaf kathodische voorbehandeling van BDD bij een negatieve potentiaal in een zuur milieu een succesvolle regeneratie van het elektrodeoppervlak. Daardoor worden zo hoog mogelijke omzettingswaarden en betrouwbare en reproduceerbare resultaten verkregen, zelfs na herhaaldelijk gebruik van de elektrode.

Toekomstperspectieven

Elektrochemische splitsing van eiwitten naast Tyr- en Trp-residuen heeft mogelijkheden voor toepassingen binnen proteomics, maar heeft nog steeds als nadeel dat opbrengsten uit splitsing laag zijn. Bovendien zijn er problemen met adsorptie aan het elektrodeoppervlak, die oxidatieopbrengsten en samenstelling van het product beïnvloedt. Concurrerende niet-splitsingsoxidatiereacties zijn moeilijk te voorkomen en verhogen de complexiteit van het verkregen peptidemengsel. Verdere methodeoptimalisatie middels chemische labeling van elektrochemisch gesplitste peptides, zoals geïntroduceerd in hoofdstuk 4 van dit proefschrift, heeft de mogelijkheid om spirolacton-bevattende tussenproducten af te vangen, te verrijken en hiermee hun detectie te vergemakkelijken.

Derivatisering met affiniteitslabels, stabiel-gelabelde isotopen of fluorescerende kleurstoffen zijn veelbelovende toekomstige strategieën. Chemische labeling van spirolactonen met biotine-affiniteitslabels is een optie voor affiniteitszuivering en verrijking van de gesplitste peptiden met avidine of streptavidine deeltjes. Als alternatief kunnen stationaire fasen die primaire amines bevatten worden gebruikt voor koppeling aan het spirolacton van het N-terminale splitsingsproduct. Tenslotte kan men het primaire amine van de stationaire fase combineren met een te splitsen spacer zoals een disulfidebinding. Beide benaderingen kunnen de complexiteit van het mengsel verminderen door het verwijderen van niet-geoxideerde of niet-gesplitste oxidatieproducten en verbeteren de detectie van elektrochemisch gesplitste peptiden en eiwitten. Andere interessante primaire-aminereagentia zijn stabiel gelabelde isotopen, die kunnen worden gebruikt voor absolute en relatieve kwantificatie en fluorescerende kleurstoffen voor gevoelige spectrofotometrische detectie. Ontwikkeling en optimalisatie van deze derivatiseringsstrategieën zouden de toepassing van de techniek voor de analyse van complexe eiwitmengsels van celkweken of biovloeistoffen kunnen ondersteunen.

Het ontwerp van elektrochemische cellen is een ander gebied dat verdere ontwikkeling en optimalisatie vereist, aangezien het significante invloed heeft op EC-reactieopbrengsten. Ons werk, gepresenteerd in hoofdstuk 5, laat adsorptieproblemen zien aan BDD-elektrodes bij het oxideren van grote peptiden en eiwitten op de traditionele koolstofelektroden. Oxidatie op BDD-elektroden kan hogere opbrengsten geven, terwijl adsorptie aanzienlijk lager is dan bij GC. De prestatie van een dergelijke electrode hangt af van de intrinsieke, fysische, chemische en elektronische eigenschappen (bijv. dopingniveau, aanwezigheid van diamantverontreinigingen en kristallijne vorm), die moeten worden geëvalueerd om het geschikte materiaal voor elektrochemische splitsing te vinden. Regeneratie van het oppervlak door kathodische voorbehandelingen is een ander belangrijk aspect om de herhaalbaarheid te waarborgen. Een andere belangrijke stap om EC te integreren in de proteomics workflow is miniaturisatie om lage monstervolumes te kunnen verwerken en de efficiëntie te verbeteren. Fabricage van microfluïdische chips, die drie-electrode EC-cellen bevatten, is voltooid en wordt met succes toegepast in metabolismestudies. Het is denkbaar dat het ontwerp van microfluïdische doorstroomchips

met geschikte werkelektrodematerialen de peptiden en eiwitten op dezelfde wijze oxideert en splitst als macroscopische EC-cellen. Bovendien biedt miniaturisatie het voordeel van verdere systeemintegratie, die kan worden gebruikt om ESI-naalden en/of LC-kolommen in de chip in te bouwen. Hierdoor kunnen gescheiden 'unit-operations' van een typische proteomics workflow op een chip[worden gecombineerd. Door post-splitsing derivatisering op te nemen in het chipsysteem zal een algemeen, volledig geïntegreerd niet-enzymatisch microfluïdisch platform voor proteomics ontstaan.

Elektrochemische splitsing bevordert het proteomicsonderzoek en eiwitanalyse in het algemeen. Het zal helpen bij de aanpak van praktische problemen bij onderzoek naar biomarkers, maar ook op het gebied van biofarmaceutica, een nieuwe klasse van veelbelovende farmaceutische producten die momenteel meer dan een derde van alle nieuwe geneesmiddelen zijn op de gehele markt. Eiwitanalyse is cruciaal in veel gebieden van de samenleving en kan grote economische impact hebben variërend van de ontdekking van diagnostische biomarkers voor ziektes tot de analyse van eiwitgeneesmiddelen, de beoordeling van de voedselveiligheid en de detectie van milieuvervuiling. Nieuwe analytische en instrumentele middelen zijn van belang om de verschillende stadia binnen de experimentele workflow te versnellen en te automatiseren. Dit geldt vooral voor bepaalde eiwitklassen, zoals transmembraaneiwitten, die momenteel de grootste groep drug-targets worden genoemd.

Acknowledgments

This is it! The smile on my face equals the relief I feel as I'm writing the last lines of my thesis. It has been a long and epic journey and making this book would not have been possible without the company, help and support of all the people around me during the last years.

First of all I would like to thank my thesis promotor Prof. Dr. Rainer Bischoff together with my co-promotors Dr. Andries Bruins and Dr. Hjalmar Permentier for giving me the opportunity to complete my PhD studies in their group. Thank you, Rainer, for guiding my research and for remaining enthusiast all the way. I am grateful for your constant input, all your advices and for the very fast corrections of my manuscripts. Andries, I am deeply honored to have learned Mass Spectrometry with one of the very best in the field. Your knowledge is endless and I was always fascinated by your enthusiasm of transmitting it to others. Thank you also for all the help with both technical and scientific issues. Thank you, Hjalmar, for assisting me on a daily basis and for helping me, in a very pragmatic way, whenever I was facing difficulties. I truly appreciated your consistent support. I wish you all the best at the head of the Mass Spectrometry Core Facility.

Thanks to the reading committee, Prof. Dr. Sabeth Verpoorte, Prof. Dr. Gerard Roelfes and Prof. Dr. Uwe Karst for accepting to spend time reading and evaluating my thesis.

Thank you to my dear paranymphs, Manfred and Jannis, for all the help organizing the defence while I was sunbathing in the west coast. Thank you also to Kanat of course! I consider myself lucky to have met people like you. You made my stay in Groningen turn into a wonderful journey and I will never forget all the times we spent together.

Special thanks to my master students Niels en Niek. I very much enjoyed supervising you guys. Niels, thank you for all the work in the lab (and I know it wasn't easy!) and for always keeping the motivation up high. I wish you both a lot of success in your future career. Many thanks also to the people at Astra Zeneca in Mölndal for the enjoyable collaboration. Ulrik and Lars, thank you for organizing the trips and for the scientific help. Thank you Gunnar for your time and help during the LC-NMR experiments. I very much enjoyed my time in beautiful Sweden!

Thanks to all members of the research groups Analytical Biochemistry, Pharmaceutical Analysis and the Mass Spectrometry Core Facility for the nice time at work. Although I was a bit isolated in the 1st floor, I enjoyed the lunches, borrels & uitjes we had together. Nicolas (lapin!) and Jan, I was glad to share your office during my first weeks in the lab. Het was kort maar krachtig. Berend, thank you so much for being my klaverjassen partner during the occasional Friday evening cards sessions in De Toeter. Pity we only ended 2nd most of the times, I think we deserved better! Jolanda, thank you very much for all your administrative help, I don't know how I could have done it without you. Thank you to the MS lab crew for all the help and the good atmosphere on a daily basis. Annie, Margot and Marcel, it was a real pleasure to have you as colleagues and seeing your good mood every day definitely cheered me up when my experiments weren't working. I wish you and your families all the best in the future. And last but not least, Eslam! It was a great pleasure to have you as an officemate although I had more the feeling that I was working in your living room. You are a talented scientist and I wish you all the best for your career!

Thank you to all my colleagues at Brains Online for the very enjoyable moments. It was not an easy time for me working and finishing-up my thesis at the same time but you all really were of great support. Big-up to the Bioanalytical department of course, it was a real pleasure to work with such a skillful and pleasant team. Robert, Wim and Harold, 1000 thanks for your valuable help translating my summary in Dutch! I am truly thankful for the time you spent on that. Carlos, I enjoyed very much all the discussions during our breaks and going to the occasional gig with you. Best of luck with the completion of your thesis, man! Corry and Gunnar, I would like once more to thank you for all the flexibility you gave me to allow me finishing my thesis. Thank you, Thomas and Arash, for giving me the opportunity to work for Brains Online LLC. I'm glad that I am able to pursue my career within the same company.

I have been lucky to meet many people from all over the world during my 6 years in Groningen and I must say this has been an incredible and enriching experience for me. I will always have a special feeling when thinking back of my time there. There are unfortunately too many people to be named and the last thing I want to do is to forget anyone. So, thank you to all of you! Special thanks however to my several housemates for being able to cope with me on a daily basis. I will never forget the container family with whom I had a terrific time during my 1st year and the peaceful moments spent in De Brink with Sandy, Nick, James and G-G! Simon,

vu que tu faisais parti des meubles à l'appart', je ne peux que te saluer aussi. Merci d'avoir réparé mes vélos quand j'étais en galère et merci pour ton amitié.

Je voudrais aussi saluer tous mes amis ainsi que ma famille toujours en France ou ailleurs pour tous les bons moments partagés lors de mes visites éclaires en Alsace ou de mes weekends ecpmiens. C'est un bon sentiment que de réaliser que notre amitié perdure malgré la distance et la faible fréquence de mes visites. Merci a vous !

Maman, papa, du fond du cœur merci pour votre inconditionnel soutien ainsi que pour tous les efforts que vous avez fait pour me permettre d'en arriver là. Merci de m'avoir toujours encouragé dans toutes mes décisions même si je sais que le fait de me voir partir n'a pas été évident. Vos valeurs et votre générosité m'ont toujours servi d'exemple et c'est en grande partie grâce à vous que je suis devenu la personne que je suis maintenant. Cette thèse je vous la dédie. J'ai aussi une grande pensée pour mes grands-parents qui ne sont malheureusement plus parmi nous et qui, je l'espère, auraient été fiers de moi. Thomas & Antoine, mes p'tis mecs! Vous ne vous imaginez pas à quel point c'est dur de devoir vous voir grandir à distance. Le mot bonheur prend tout son sens quand j'ai la chance de passer du temps avec vous et vous étiez sans le savoir un des meilleurs remèdes lors des périodes difficiles de ma thèse. Même si la route est encore longue, je vous souhaite de tout cœur d'avoir autant d'opportunités que j'ai eu la chance d'avoir et je sais que vos parents vont tout faire pour vous aider à trouver votre voie. Aurélie et David, merci aussi pour tout votre soutien et votre aide. Les mots me manquent pour décrire a quel point vous êtes formidables mais sachez que vous êtes de véritables exemples pour moi.

Mes tous derniers mots s'adressent à toi Jéjé, mon petit frère! Tu m'as montré la voie il y a quelques années de ça (déjà) et tes conseils et encouragements ont énormement contribué à ce que je puisse finalement finir cette thèse. Merci pour toujours avoir sû trouver les mots qu'il faut quand il le fallait. Les aléas de la vie font que je m'éloigne une fois de plus de toi mais le jour viendra où on se rapprochera à nouveau et crois-moi, j'ai hâte! Tu es le plus beau cadeau que la vie m'a offert. Merci pour qui tu es et pour tout ce que tu représentes pour moi. Je suis fier de toi. A mol !

San Francisco, February 2013

Julien